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IDENTIFICATION AND CHARACTERIZATION OF MAREK'S DISEASE VIRUS UNIQUE SHORT REGION GENES AND THEIR PRODUCTS

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IDENTIFICATION AND CHARACTERIZATION OF MAREK'S DISEASE VIRUS UNIQUE SHORT REGION GENES AND THEIR PRODUCTS

By

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ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF MAREK'S DISEASE VIRUS UNIQUE SHORT REGION GENES AND THEIR PRODUCTS

By

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Because of its lymphotropic properties, Marek's disease virus (MDV) has been classified as a gammaherpesvirus. However, its genome structure most closely resembles that of the alphaherpesvirus prototype, herpes simplex virus (HSV). To examine the nature of this discrepancy, an analysis of MDV's unique short (U_S) region was undertaken. Alphaherpesvirus U_S regions exhibit significant evolutionary divergence, encoding genes specific to members of this phylogenetically related herpesvirus lineage. This includes a cluster of glycoprotein genes possibly conserved in MDV. Attempts to identify such genes of potential importance to pathogenesis and immunoprotection, led to the complete nucleotide sequence determination of MDV's 11,286 base pair U_S region (pathogenic GA strain). Sequence analysis identified 7 alphaherpesvirus related genes, including MDV counterparts of HSV US1, -2, and -3; US6, -7, and -8 (glycoproteins gD, gI, and gE, respectively) and US10. In addition, three MDV-specific ORFs and a novel fowlpox virus-related ORF were identified. These results confirm and extend upon recently published data which demonstrate a closer phylogenetic relationship between MDV and alphaherpesviruses.

MDV U_S region polypeptides were analyzed with monospecific, polyclonal antisera generated from a panel of 16 different bacterially-expressed

trpE fusion protein immunogens representing 9 of the 11 ORFs. The resulting antibodies were found to immunoprecipitate 6 of the 7 alphaherpesvirus-related MDV homologs from infected avian cell cultures. MDV US1 was found to express an unusual 27,24-kDa late class cytoplasmic phosphoprotein, in contrast to its larger 68-kDa immediate-early nuclear phosphoprotein counterpart, HSV ICP22. US2 and -10 were identified as 30- and 24-kDa polypeptides, respectively. Antisera directed against three different regions of the protein kinase-related US3 gene were all found to precipitate a 47,49-kDa doublet; one of these was found to specifically react with a 68-kDa cellular protein as well. Like other gI/gE-related products, MDV's counterparts were found to coprecipitate together. Antibodies reactive with the gD epitopes of three different bacterially-expressed fusion proteins failed to precipitate gD from avian cell cultures. Together, these results raise a number of interesting questions which highlight the potential importance of MDV U_S region genes in determining many of MDV's unusual biological properties.

More precise information on genetic relatedness awaits resolution of methods to measure nucleotide sequences of the DNA of herpesviruses.

Andre J. Nahmias, 1972.

Nucleotide sequence data are necessarily the basis of the taxonomy of the family *Herpesviridae*. The central issue is the identification of the correlates which must be culled from such data for a truly useful taxonomy.

Herpesvirus Study Group, International Committee on Taxonomy of Viruses, 1992.

To my dear Lelda, without whose love and support this could not have been possible...

To my wife's parents, Maiga and Auseklis, whose emotional and material support carried us through many difficult times...

and

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I. Introduction to herpesviruses.

A. Historical aspects.

Although the concept of herpes as a disease has existed for at least 25 centuries, its meaning, nomenclature and description has changed considerably (Wildy, 1973). Modern views of herpes were largely derived from the definitions and work of Wilan and Bateman (1814). Herpes simplex was shown to be an infectious agent in 1873, herpes zoster (varicella zoster) in 1909. Jozsef Marek was the first to recognize Marek's disease in domestic fowl, although he could not ascertain the cause (Marek, 1907).

Despite the clinical observations associated with these agents, a conceptual understanding of viruses was largely unknown prior to 1957. The introduction of negative staining in 1959 marked the the turning point in virus classification (Wildy, 1973). This technique enabled the full exploitation of electron microscopy to reveal fine differences in virus particles. In conjunction with biochemical virological techniques for distinguishing DNA from RNA, morphological criteria for virus taxonomy soon took hold. A unifying description of herpesviruses was soon developed. All were found to share a characteristic morphology conforming to the following description: 'Viruses of eukaryotes with linear, double-stranded DNA genomes of more than $80x10^6$ mol. wt. which are replicated in the nucleus of infected cells, assembled into 100 nm diam. icosahedral capsids composed of 162 prismatic capsomeres which are enclosed in glycoprotein and lipid (ether-sensitive) envelopes to give the normally infectious extracellular form of the virus' (Honess and Watson, 1977). Beyond

this general description, however, exists a large family of viruses with an extraordinary range of biological potentials.

B. Characteristics and diversity of herpesviruses.

More than 80 distinct herpesviruses have been isolated. Herpesviruses have been isolated from virtually every species examined. This includes hosts as diverse as fish, amphibians, reptiles, birds, and numerous mammals (Nahmias, 1972). A single host may be inhabited by multiple herpesviruses with uniquely different patterns of infection and pathogenesis. Herpesviruses exhibit greatly variable tissue tropisms, but are generally restricted in nature to a particular host or a few closely related species. Once infected, a host will usually harbor the virus for life. This aspect of herpesvirus biology, latency, is one of its most distinguishing features. Under this condition, the virus can periodically reactivate and infect new hosts. In its natural host, beyond the newborn age, herpesviruses are generally quite harmless; however, newborn or immunocompromised individuals generally exhibit a wide range of pathological manifestations. Despite their often inocuous nature, herpesviruses can be extremely dangerous when crossing their normal host-species barrier. Together, these aspects reflect a long evolutionary history characterized by the selection for isolates which live in relative harmony with their hosts.

C. The human herpesviruses.

Because of their clinical significance to man, human herpesviruses have been the most studied. Presently, there are seven distinct herpesviruses identified in humans; herpes simplex virus types 1 and 2 (HSV-1, HSV-2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), and human herpesvirus types 6 and 7 (HHV-6, HHV-7). Considering their often inocuous nature, it shouldn't be a surprise if more were identified in the future. HHV-7 wasn't recognized until 1990 (Frenkel et al., 1990). Most

adults are seropositive for several of these herpesviruses. In many cases, human herpesvirus infections are subclinical in nature. However, in neonates or immunocompromised adults viremias are frequently observed and often associated with deadly consequences. Infections in immunocompromised adults highlight the ability of herpesviruses to persist in a latent state, generally held in check by the host's immune system. Immunosuppressive therapies for cancer or bone marrow transplantations often provide an opportunity for these viruses to reactivate and cause a severe, acute infection. In noncompromised hosts over one month of age, viremias are generally considered to be absent. However, this view may have to be reconsidered, viremias appear to occur rather routinely in immunocompetent children with varicella (Ozaki et al., 1986).

II. Introduction to Marek's disease virus.

A. General pathogenic characteristics.

Marek's disease virus (MDV) is a strongly cell-associated, acutely transforming herpesvirus which can rapidly (3 weeks) induce T-cell lymphomas in chickens (for a recent review, see Calnek and Witter, 1991). While Marek's disease (MD) is largely regarded as a useful natural host model for oncogenesis, other studies highlight the importance of MDV as a useful natural host model for atherosclerosis (Fabricant, 1985) and the Guillain-Barre syndrome (Pepose et al., 1981). Various isolates have been reported to cause lymphoproliferative neural lesions, peripheral nerve demyelination, blindness. and paralysis.

B. MD as a model for tumor immunity studies.

Because of the protection afforded against MDV by vaccination (Churchill et al., 1969; Okazaki et al., 1970), the MD system has long been considered as a useful natural host model for tumor immunity studies. MD tumors can be prevented by vaccination with cell-culture-attenuated pathogenic

strains and serologically-related naturally apathogenic strains, including herpesvirus of turkeys (HVT). Although the mechanism for immunoprotection is far from clear, studies have implicated both anti-viral- and anti-tumor immunity (Powell and Rowell, 1977).

C. Serotypes and pathotypes associated with the MD system.

On the basis of immunological criteria serologically-related strains of MDV- and HVT have been assigned to three serotype groups: serotype 1, represent pathogenic strains of MDV and their cell-culture-attenuated variants; serotype 2, represent naturally apathogenic MDVs; and serotype 3, HVT. These three serotypes can be distinguished by their reactivity with serotype-specific monoclonal antibodies (Lee et al., 1983). In addition, these serotypes exhibit uniquely distinct restriction digestion patterns. Taken together, the three 'MDV' serotypes represent antigenically-related, yet phylogenetically-distinct virus entities.

The three MDV serotypes are characterized by a range of pathotypes according to their virulence and pathogenicity in susceptible chicks. These include *highly pathogenic* (acute) strains, causing a high incidence of visceral and neural lymphomatosis; *moderately pathogenic* (classic) strains, causing primarily neural lesions, often at a lower incidence; *and mildly pathogenic* or *nonpathogenic* strains causing no gross lesions (Payne, 1982).

For many years MDVs were primarily known to induce a classical, neuropathologic disease. Beginning with the late 1950's, more virulent, oncogenic strains began to replace the less virulent, neuropathologic strains as the predominant field isolates responsible for disease. Thus, an evolutionary pattern for virulence has been recognized, characterized by selective pressures for continually increased virulence (Calnek and Witter, 1991). This has led to a further differentiation of serotype 1 isolates according to their pathogenicity in

both vaccinated and unvaccinated chicks (Witter, 1983). The so-called *very virulent* (vvMDV) isolates (e.g. Md11, RB1B) represent highly oncogenic serotype 1 strains that are often refractory to traditional HVT vaccines (e.g. less than 77% protection). These strains are largely responsible for vaccine failures in the field. In contrast, better protection has been achieved against *virulent* (vMDV) serotype 1 strains (greater than 77% protection). These are represented by common oncogenic strains, such as GA, JM, and HPRS-16. As a group, vvMDVs are more pathogenic and viscerotropic than vMDVs.

One strategy for protection against vvMDV strains is based on the observation that a combination of vaccine viruses provide better protection than the individual strains alone (Witter, 1982). This phenomenon has been referred to as *protective synergism*. Further studies have indicated that serotype 2- and 3 combinations provide better protection than serotype 1 (attenuated) and 3, or 1- and 2 combinations (Witter, 1987). Ideally, a vaccine virus should be attenuated for pathogenicity, yet able to replicate efficiently in birds. Since cell-culture-attenuation often leads to decreased replication in birds, an optimal balance between attenuation and in vivo replication must be achieved. This can be accomplished by alternating MDV passages between both cell-cultures and birds. The former selects for more attenuated strains, the latter for more pathogenic, better replicating strains. Recent strategies have employed this approach to develop vaccine strains derived from vvMDV isolates in order to achieve better protection against their more pathogenic relatives (Witter, 1991).

D. Natural transmission.

MDV has been considered as the only known oncogenic virus with a highly efficient mode of horizontal transmission in nature (Klein, 1972). This transmission is mediated by the maturation and release of virus from infected feather follicle epithelial (FFE) cells, usually by an air-borne route involving

poultry house dust and chicken dander. Like papillomaviruses, which are dependent on highly differentiated epithelial cells for expression of structural proteins and formation of virions, production of fully enveloped MDV virions can only take place in highly differentiated epithelial cells (Calnek et al., 1970; Nazerian and Witter, 1970). Although the resulting virions are fully infectious in a cell-free form, release of these virions from FFE cells has never been observed. Based on the evidence below (Carozza et al., 1973), a compelling case can be made for *cell-associated* spread of MDV in horizontal transmission. Herein appears to lie the secret to MDV's efficient transmission. In contrast to cell-free virus from poultry dust sediments, which loses its infectivity in less than two weeks, intact poultry dust carrying desquamated MDV-containing FFE cells retains its infectivity for at least 205 days at ambient room temperatures (and much longer at lower temperatures) (Carozza et al., 1973). Unlike many other herpesviruses, MDV cannot be transmitted in a vertical fashion.

E. Host range.

Natural infection with MDV is known to primarily affect members of the genus *Gallus*, although it has been described in other birds of the *Galliformes* order, including turkey and quail (Biggs, 1985). MDV has been isolated from naturally infected quail with lymphoproliferative tumors affecting many visceral organs; however, infected quail usually lack the peripheral nerve lesions characteristic of MD in chickens (Imai et al., 1990). A milder infection has been noted in jungle fowl, both feral and zoological (Biggs, 1985). Despite possible questions regarding the natural host from which MDV evolved throughout most of its existence, chickens are clearly the most important 'natural host' in an economic sense.

F. Growth of MDV *in vitro*.

1. Cell cultures.

Unlike many other herpesviruses, especially other alphaherpesviruses, MDV has a very limited host range *in vitro*. Growth is primarily restricted to primary avian cell cultures. The major difficulty in working with MDV is its slowly progressing, tightly cell-associated nature. This precludes straightforward plaque purification of virus, as well as the establishment of one-step growth conditions for effective temporal gene regulation studies. Cell culture systems for producing enveloped, cell-free virus are currently unavailable. To obtain sufficient quantities of material with which to work, it is necessary to passage infected cells onto uninfected cell monolayers. Even though cell-free infectious virus can be purified from FFE cells, titers are at best limited to 10^4 PFU/ml.

Based on optimal growth properties, serotype 1 strains are usually grown in duck embryo fibroblast (DEF) cultures; serotypes 2- and 3 are most often grown in chick embryo fibroblast (CEF) cultures. Chick kidney- and lymphocyte (Calnek et al., 1982) cultures have been used as well. The differential growth characteristics in primary cultures can be used to differentiate between pathogenic and non-pathogenic serotypes (Cho, 1976a, 1976b). Furthermore, in contrast to MDV, which normally fails to grow in continuous cell lines, HVT is distinguished by its ability to grow in the quail fibroblast cell line, QT-35 (Cho, 1981).

2. Lymphoblastoid cell lines.

Various T-lymphoblastoid cell lines have been established from MD tumors. Most of these are considered *producer* cell lines (e.g. MSB-1), which can be induced to produce virus following co-cultivation onto fibroblast- or kidney cell monolayers or following inoculation in birds. *Non-*

producer lines (e.g. MDCC-RP1) are those from which virus cannot be rescued. The latter generally fail to produce antigens detectable with conventional anti-MDV seras. However, treatment with 5-iodo-2-deoxyuridine (IUDR) can lead to the detection of a phosphorylated polypeptide (pp38) using a pp38-specific monoclonal antibody. Producer cell lines can be further characterized as *expression* or *non-expression* cell lines. The former contain a high proportion of cells that spontaneously produce immunologically detectable antigens; the latter contain few such cells and generally require IUDR treatment in order to produce detectable levels of antigen. Two groups have recently succeeded in establishing lymphoblastoid cell lines following *in vitro* infection (Ikuta et al., 1987; Calnek and Schat, 1991).

G. Sequential events in pathogenesis.

In antibody-free, genetically susceptible chickens infected with oncogenic strains of MDV, a sequential pattern of events has been recognized which accounts for the establishment of lymphomas and death. This sequence involves four phases: (1) an early cytolytic infection; (2) a latent infection; (3) immunosuppression in conjunction with a secondary cytolytic infection, and (4) oncogenic transformation (Calnek, 1986).

1. Early cytolytic infection.

MDV generally gains entry into chickens by way of the respiratory tract. This is probably the only place in which cell-free entry of virus can occur. From here the virus makes its way into the lymphoid system, where an early necrotizing infection is detectable as early as 1-2 days postinfection (DPI). In lymphoid- and other tissues (except the FFE), infections are cell-associated and productive-restrictive; few or no enveloped virions are produced. Lymphoid infections involve the three major lymphoid organs (e.g. the spleen, thymus and bursa of Fabricius) and result in cell death and atrophy

to the bursa and thymus; consequently, a temporary immunosuppression develops. The infection peaks at 4-5 DPI and subsides by 6-7 DPI after which viral antigens become temporarily undetectable. Bursa-derived B-cells are the predominant target for this early phase of the infection.

2. Latent infection.

The onset of an immune response beginning at 5-7 DPI coincides with two important events. A drop-off in the cytolytic infection occurs and a cell-mediated response leads to the activation of T-cells which begin to express the Ia or class II MHC antigen. The activated T-cells then become permissive for MDV, probably as a result of their interaction with the cytolytically-infected B cells. The MDV-infected Ia-bearing (class II MHC) T-cells represent the primary target for the ensuing latent infection. Little or no gene expression occurs during this phase. These latently-infected cells are thought to persist throughout the life of the bird (Witter et al., 1971) and can be activated into lytic growth by cocultivation onto primary fibroblast cell monolayers. Cytolytically- and/or latently-infected lymphoid cells are thought to seed the various epithelial tissues prior to a secondary cytolytic infection which begins after the 2nd or 3rd week post-infection.

3. Immunosuppression and secondary cytolytic infection.

It is thought that immunocompetence is required for the establishment and maintenance of latency (Buscaglia et al., 1988). This appears to be mediated by a latency-maintenance factor (LMF) found in the conditioned media of latently-infected cells (Buscaglia and Calnek, 1988). For reasons that are not yet known, some birds undergo an immunosuppression which leads to a release from latency. A secondary cytolytic infection follows, not only in epithelial tissues (such as the FFE), but in the lymphoid cells as well. Although chickens genetically susceptible- or resistant to transformation by MDV are both

subject to the early cytolytic infection, late cytolytic lymphoid infections are generally restricted to genetically-susceptible birds. Whatever the reason, an amplification of infected T-lymphocytes results; this large pool of infected cells ultimately serves as the reservoir for oncogenic transformation which follows.

4. Oncogenic transformation.

Lymphomas can develop as early as three weeks post-infection. There is no clear consensus regarding the type of T-cell subset permissive for transformation. Most cell lines derived from normally occurring tumors have been found to be CD4+ CD8-. However, 45% of the cell lines derived from experimentally-infected local lesions were found to be CD4- CD8+, 34% CD4- CD8-, and only 21% CD4+ CD8- (Schat et al., 1991). On the other hand, all have been observed to express Ia antigen, CD3 and/or TCR. Thus, activation of T-cells appears to be a consequence of MDV infection and a prerequisite for transformation.

Tumor induction is determined by the complex interplay of a number of factors, including (i) innate oncogenic and immunosuppressive potential associated with a given MDV isolate, and (ii) differences in genetic resistance dictated by factors such as age, sex and host genotype (Calnek and Witter, 1991). Genetic resistance to MD appears to be controlled by MHC-dependent or -independent mechanisms. Non-MHC-controlled resistance in line 6 chickens has been characterized by a reduced susceptibility to virus. This could be attributed to T-cell targets with fewer virus receptors, receptors of lower affinity for virus, or fewer target cells with appropriate receptors (Gallatin and Longnecker, 1979).

At the present time, nothing is known about the genes responsible for transformation. Most transcriptional activity appears to reside in the inverted repeat regions, particularly the inverted repeat long (IR_L) region. However, the

possible involvement of unique short (U_S) region can not yet be ruled out (Schat et al., 1989). Various studies (Bradley et al., 1989a; Chen and Velicer, 1991) have focused on MDV transcription near an area of the IR_L containing a 132-bp repeat element whose copy number is significantly increased in attenuated MDV strains (Maotani et al., 1986; Silva and Witter, 1985). One group has reported that the 132-bp expansion causes the premature termination of a putative tumorigenicity-related transcript (Bradley et al., 1989b) which contains two small open reading frames (Iwata et al., 1992).

The significance of the above findings are not yet clear. The putative tumorigenicity-associated transcripts are similarly expressed during lytic infections as well; such expression may be incompatible with a transformed state that requires repression of functions associated with lytic infections. In addition, a number of these studies have relied on iodo-deoxyuridine (IUdR) to artificially induce viral gene expression in MD tumor cell lines; this raises questions concerning the relevance of the accompanying gene expression with regard to transformation. While a great deal of attention has focused on changes in gene expression concommitant with attenuation, rather than affecting the expression of tumorigenicity-associated genes, the changes may simply reflect in vitro selection pressures that have altered the infectivity of oncogenic strains for T-cells (Schat et al., 1985).

Nevertheless, further studies involving the IR_L region are clearly warranted, inasmuch as IR_L-specific gene expression has been consistently observed in natural tumors and tumor cell lines (Schat et al., 1989; Sugaya et al., 1990). Antisense oligonucleotides specific for MDV IR_L sequences have recently been reported to arrest the growth of an MDV-transformed cell line (Kawamura et al., 1991). Two groups have recently mapped and sequenced a gene upstream of the 132-bp repeat (Cui et al., 1991; Chen and Velicer et al.,

1992) which was found to express a 38-kDa phosphoprotein previously detected in tumor cell lines using a monoclonal antibody (Ikuta et al., 1985; Nakajima et al., 1987). Jones et al. (1992) have recently identified a gene located downstream of the 132-bp repeat which is abundantly expressed in tumor cells. Sequence analysis of this gene, designated as *meq*, led to the identification of N-terminal proline, basic, and leucine zipper regions with the same spacing and characteristics shared by members of the jun/fos family. Together with a large proline rich region containing a novel repeat motif rich in proline, serine, threonine, and acidic amino acids (aa) at its C-terminal end, these motifs suggest a role in transcriptional activation.

III. Herpesvirus classification.

A. Early attempts.

By comparing B virus with pseudorabies virus, Sabin (1934) provided the first evidence for biologic- and serologic relatedness between two herpesviruses. (Wildy, 1973). By 1952 the herpesvirus group grew to include (in addition to B virus and pseudorabies) herpes simplex, varicella, and zoster. At the time it was not yet known that the latter two were one and the same. Using morphological criteria, an increasing number of herpesviruses began to be identified in the early 1960's.

In one of the first attempts to classify herpesviruses, Melnick et al. (1964) proposed a herpesvirus classification scheme based on in vitro growth properties. *Group A* included viruses readily released from cells in active form; *Group B* included avidly cell-associated viruses releasing little, if any, cell-free virus. The former was represented (among others) by herpes simplex virus, B virus, and pseudorabies virus; the latter, varicella-zoster- and cytomegalo-viruses of man.

B. Early views of MDV and the development of taxonomic criteria.

Since the 1960's, classification schemes have had an important impact on the way we view herpesviruses. In 1969, soon after MDV was determined to be the causative agent of lymphoid tumors in MD, studies of its DNA composition and in vitro growth properties led Lee et al. (1969) to suggest the possibility that MDV was a cytomegalovirus. Even to this day such an example illustrates that taxonomy is an evolving process, largely dictated by the technology and popular views of its day. At that time, proportional analyses of guanosine and cytosine (G + C) contents was a popular endeavor. This reflected its recently recognized value (in other systems) as an indicator of gross sequence relationships (Sueoka, 1961). Of disappointment to taxonomists was the subsequent observation that herpesviruses possessed an unprecedented range of mean nucleotide compositions lacking any sort of consistency with other objective criteria, such as serological relatedness or similarities in host range (Honess and Watson, 1977).

Beginning with the 1970's, other objective criteria began to gain attention. One of these was DNA-DNA hybridization. It is somewhat of an irony that one of the major proponents of the current biologically-based classification system (B. Roizman), as early as 1972, led a study to examine whether viruses associated with neoplasia share common physical and genetic properties differentiating them from other herpesviruses. Its basis rested on the premise that 'superficial' aspects of biological properties are ultimately determined by 'the information content of the virus' (Bachenheimer, 1972). Interestingly enough, this question was addressed by focusing on the comparative genetic properties of MDV and HSV. Although HSV-1 and -2 were found to exhibit 40% homology by DNA-DNA hybridization, the extent of homology between MDV and HSV-1 or HSV-2 was found to be <1-2% respectively. Despite what may

privately have been a disappointment to Roizman (considering all the discussions about herpesviruses and cancer at that time, see Roizman, 1972 for instance), as a consolation, the study did find that G+C compositions among herpesviruses associated with neoplasia did not differ significantly from those of other herpesviruses. These results appeared to represent a foreshadowing of events to come.

Not long before the 1971 'Oncogenesis and Herpesviruses' symposium held in Cambridge, England (at which the above results were presented), Epstein had reported the discovery and in vitro growth adaptation of a herpes-type virus (EB virus) found in Burkitt's lymphoma tumor cells (Epstein et al., 1964) which was later found to be antigenically distinct from HSV, VZV, and HCMV (Hummeler et al., 1966). Poor growth properties and a tropism for lymphocytes appears to have raised interest in a possible relation between this new virus and MDV. Using a 'state-of-the-art' DNA-DNA hybridization protocol, zur Hausen and colleagues (1970) were unable to demonstrate significant homology between MDV tumor DNA (or HSV-and human cytomegalovirus-infected cell lines) and the DNA present in the Burkitt's lymphoma lines. Considering the stringent hybridization conditions of that day, and the differences in homology between herpesviruses at the nucleic acid level that we recognize today, such a result (and that of Bachenheimer et al, 1972, above) should not be surprising. Nevertheless, other investigators continued to search for a serological relationship between MDV and EBV (Ono et al., 1970; Kato et al., 1972; Ross et al., 1972). It is a little ironic that the latter study did in fact demonstrate a significant serological relationship between MDV and PRV.

C. Concerted attempts to name and classify herpesviruses.

In spite of attempts to characterize and classify herpesviruses by objective criteria which employ serologic approaches (cross-neutralization,

complement fixation, immunodiffusion, immunofluorescence, particle agglutination) and comparative 'high-resolution' polyacrylamide gel electrophoresis (Honess and Watson, 1977), methodological limitations precluded an accurate and definitive assessment of phylogenetic relatedness. This led to a more subjective approach based on the recognition of biologically-shared properties. Such an approach began to take form in 1977, six years following the establishment (in 1971) of a Herpesvirus Study Group appointed by the International Commission for the Nomenclature of Viruses (ICNV) to make recommendations concerning the nomenclature and classification of herpesviruses.

1. Nomenclature.

The problem of nomenclature was first discussed at the 1971 Oncogenesis and Herpesviruses symposium (Pereira, 1972). Although it was deemed too early to try to classify herpesviruses, it was apparent that a unifying nomenclature system was needed to ward off the confusion that was likely to result from the rapid identification of new herpesvirus members. B. Roizman stated that the current nomenclature was unsatisfactory, since herpesviruses were being named at random according to the host species they infect, the disease they produce or their discoverers. This brought forth a proposal illustrating some of the nauseating and somewhat humorous aspects occasionally associated with virus nomenclature issues.

The outlined proposal (Pereira, 1972) suggested a Latinized binomial nomenclature. MDV and HVT would be Herpesvirus galli 1 subspecies 1 and 2, respectively. It was suggested that the latinized forms would be used only for *formal* occasions, but that for *common* usage, anglicized forms could be adopted. People were informed to keep in mind the rules of the ICNV, which specified (among others) that neither personal, nor nonsense names could be

used, nor could the code of bacterial nomenclature be applied to viruses. One problem, voiced by Peter Wildy, was that it would be unwise to use Latin names as part of a temporary classification as such names tend to become permanent, thus causing potential difficulties in the future.

Two years later, in 1973, the Herpes Study Group agreed on a non-Latinized, Anglican-based nomenclature system (Roizman et al., 1973). Thus, MDV and HVT now became phasianid herpesvirus 2 and turkey herpesvirus 1, respectively. B virus became Cercopithecid herpesvirus 1. The currently established nomenclature also recognizes a set of formal and common names. It is probably not a surprise that the common names continue to dominate the current literature.

2. Herpesvirus subclassification.

In 1977, the Herpesvirus Study Group of the ICNV proposed a provisional classification of herpesviruses based on biological properties. The most often used classification, based on Melnick's cell-associatedness scheme, was effectively done away with, since it was argued that cell-associatedness appeared to be in conflict with known *in vivo* properties of the virus. Moreover, others had criticized this system as being overly subjective, inasmuch as herpesviruses exhibited a spectrum of 'efficiency of behaviour in tissue culture', with no clear breaks in the gradation of efficiency observed (Plummer, 1967). For the 1977 proposal, it was argued that even though phasianid herpesvirus 2 (MDV) was strongly cell-associated in cell culture its spread in nature occurred by dissemination of infectious virus rather than infected cells. As pointed out in II.D., the latter is actually more representative of the truth.

This new proposal outlined a basic framework for assigning members of the Herpesviridae into three subfamily groups: *Alphaherpesvirinae, Betaherpesvirinae,* and *Gammaherpesvirinae.* These were ordered on the basis

of the historical precedence of their prototypes, herpes simplex 1, human cytomegalovirus, and Epstein-Barr virus, respectively. Each of these three subfamilies were characterized by a set of biologically-shared properties characterized by factors such as host range, cell tropism, duration of reproductive cycle, cytopathology in cell cultures, and latent infections.

Alphaherpesviruses were characterized as possessing a variable host range, from very wide to very narrow; highly cytopathic, with a short replicative cycle; and frequently latent in ganglia. Betaherpesviruses were characterized by a narrow in vivo host range, usually restricted to growth in fibroblasts in vitro; a slow reproductive cycle; slowly progressing foci in cell cultures with enlargement of infected cells (in vivo and in vitro); inclusion bodies present in both the nucleus and cytoplasm; and latent infections frequently in salivary glands and/or other tissues. Gammaherpesviruses were characterized by a narrow host range in vivo, usually limited to the same order as the host it naturally infects; replication in lymphoblastoid cells; some growing lytically in epithelioid and fibroblastoid cells; a variable cytopathology and variable-duration replication cycle; a particular specificity for B- or T-lymphocytes with infections frequently arrested at a prelytic stage, with persistence and a minimum level of gene expression, or at a postlytic stage, causing death of the cell without production of complete virions; latent infections frequently residing in the lymphoid tissues.

Four years later, the above proposal was formally outlined in more precise detail (Roizman et al., 1981). However, the classification criteria described above were left unchanged. Also presented (at a time when sequence information was only beginning to be generated) was the rationale for this proposal. Biological properties were chosen as the dominant criterion, primarily because of practical considerations. In spite of their subjective nature,

biological properties were emphasized, since they could be most readily established following the isolation of a new herpesvirus. Phylogenetic relationships, based on genome structure, sequence homology, and/or serological relationships were to assume a secondary role in further classifying subfamily members into genera. Because many of these relationships had been fairly well established over many years of work, it would appear that an inherent bias was already present when biological criteria were developed.

D. Discrepancies between biologic- and phylogenetic properties in herpesvirus classification.

The current biologically-based herpesvirus classification system (Roizman et al., 1981) initially gained its strength from the fact that biologic properties could be readily established following isolation of new herpesviruses. This classification system has turned out to be generally consistent with genetic data that have accumulated over the past ten years or so. However, recent years have seen a downturn in the number of new herpesviruses characterized. More importantly, a number of discrepancies with this system have begun to emerge.

1. MDV.

Because of similar biological properties, especially their lymphotropism, MDV and HVT have been classified as gammaherpesviruses (Roizman et al., 1981). Members of this subfamily include, among others, EBV, a human B-lymphotropic herpesvirus, and herpesvirus saimiri (HVS), a T-lymphotropic herpesvirus of new world monkeys and lower vertebrates. Despite certain structural differences between EBV and HVS, the genomes of these and other gammaherpesviruses encode serologically-related proteins and share a common organization of coding sequences which differs from that of the neurotropic alphaherpesviruses (Davison and Taylor, 1987; Efstathiou et al.,

1990; Gompels et al., 1988; Nicholas et al., 1992b). This latter subfamily includes (among others) the human herpesviruses HSV and VZV, porcine pseudorabies virus (PRV), bovine herpesvirus (BHV) and equine herpesvirus (EHV).

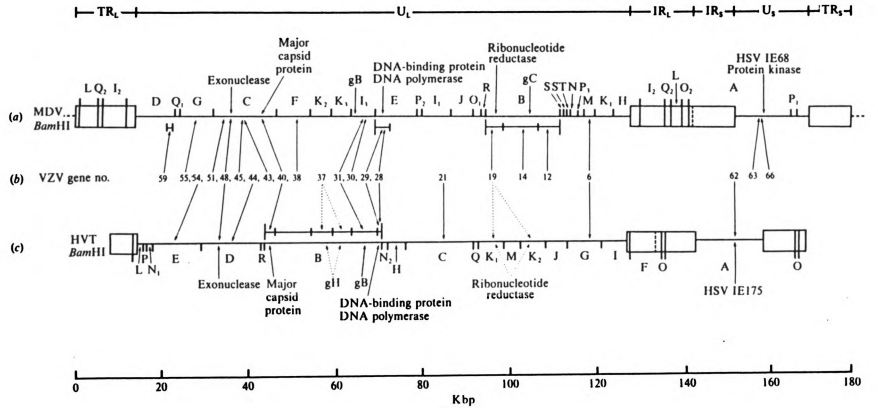
In contrast to other gammaherpesviruses, MDV and HVT have genome structures closely resembling that of the alphaherpesviruses (Cebrian et al., 1982; Fukuchi et al., 1985; Igarashi et al., 1987). The latter possess similar genome structures consisting of covalently joined long (L) and short (S) components. The S components comprise a unique segment (U_S) flanked by a pair of extensive inverted repeat regions (i.e. IR_S/TR_S or R_S). L components contain a unique segment (U_L) that *may or may not* be flanked by similarly extensive repeat regions (i.e. TR_L/IR_L). While MDV, HVT and HSV contain extensive U_L-flanking repeat sequences, VZV, PRV, BHV, and EHV do not. Thus, the genome structures of MDV and HVT most closely resemble that of HSV (Cebrian et al., 1982).

Buckmaster et al., (1988) have recently reported that the gammaherpesviruses MDV and HVT bear greater genetic similarity to alphaherpesviruses than gammaherpesviruses. This was based on analyses of numerous, randomly isolated MDV and HVT clones at the predicted aa level; not only were individual sequences found to exhibit greater relatedness to genes of HSV/VZV than EBV, but the MDV/HVT genomes were found to be generally collinear with VZV, at least with respect to the U_L region (Figure 1). Alphaherpesvirus U_S- and other S region genes originate from an area specific to members of this taxonomic subfamily, arguably their most divergent coding region. Two groups have recently confirmed the earlier proposal of Buckmaster (1988) by demonstrating extensive colinearity of alphaherpesvirus-homologous genes in the U_S regions of MDV (Brunovskis and Velicer, 1992a; Ross et al.,

FIGURE 1. Map positions of MDV and HVT genes.

*Bam*HI linkage maps of MDV and HVT are shown in (*a*) and (*c*) respectively. All VZV genes for which homologues were identified and mapped are shown in (*b*), and the positions of genes with known functions are also indicated in (*a*) and (*c*). Some MDV genes were further mapped to *Sma*I fragments (|----|) as shown in (a); for HVT genes mapping within *Bam*HI-B the gene order within that fragment was determined by further mapping to MDV *Bam*HI fragments (|----|) as shown in (*c*). The dotted lines showing the map positions of the gH gene and the ribonucleotide reductase gene in HVT indicates that in each case the precise gene location has not been resolved between two restriction fragments of the same size. From Buckmaster et al., 1988.

MAP POSITIONS OF MDV AND HVT GENES



1991). Together these results show that, in spite of their biological properties, MDV and HVT bear a much closer phylogenetic relationhip to alphaherpesviruses than gammaherpesviruses.

2. Human herpesvirus 6.

Human herpesvirus 6 (HHV-6) is a novel herpesvirus independently isolated by several groups from patients with lymphocytic disorders. HHV-6 was initially named human B lymphotropic virus because it was found to infect B lymphocytes (Salahuddin et al., 1986). However, recent studies have shown that it is primarily tropic for CD4+ T-lymphocytes (Takahashi et al., 1989) where it may act as a cofactor in AIDS (Ensoli et al., 1989). Because of its lymphotropism, Lopez et al., (1988) suggested a provisional gammaherpesvirus classification. Genetic evidence based on a 6-base-pair $(GGTTA)_n$ repeat sequence identity between MDV and HHV-6 was further interpreted as warranting such a classification (Kishi et al., 1988). In contrast to these proposals, recent sequence analysis has established a much closer phylogenetic relationship between HHV-6 and betaherpesviruses, characterized by its prototype human cytomegalovirus (Lawrence et al., 1990).

3. Channel catfish virus.

Channel catfish virus (CCV) is a relatively uncharacterized herpesvirus with an unusual genome structure packaged in a virion that is morphologically indistinguishable from those infecting higher vertebrates (Wolf and Darlington, 1971). However, in contrast to its alphaherpesvirus classification, sequence analysis of the 134,226-bp CCV genome has shown that its genetic properties are entirely distinct from those of either of the three herpesvirus subfamilies (Davison, 1992). Even though CCV possesses several characteristics common to herpesviruses, this new data suggests that CCV is representative of an entirely novel herpesvirus (sub)family.

E. Problems with the biologically-based classification system.

1. Theoretical implications.

The above discrepancies reflect a problem that may be more than simply academic. To its credit, the current biologically-based classification system has been remarkably consistent with the genetic data. It has been recently described as simple, fortuitously appropriate and defective (Roizman, 1990b). A further criticism relates to is subjectivity and potential to adversely affect our view of herpesviruses. Herpesvirus biology is exceedingly complex, perhaps more so than originally thought.

Since MDV has been regarded a gammaherpesvirus, much of the previous work interpreting MDV's properties has proceeded by analogy with the association between EBV and B cells (Wen et al., 1988, for example). MDV actually has little in common with gammaherpesviruses other than its potential to latently infect and transform lymphocytes. Because of its closer relatedness to alphaherpesviruses, it would appear that MDV's seemingly divergent biological properties are unlikely to be determined by macromolecular structures homologous to those of gammaherpesviruses (Lawrence et al., 1990). This suggests a renewed emphasis focusing on phylogenetic differences between MDV and other alphaherpesviruses as an approach for examining the molecular basis of the divergent biological properties.

One of the chief difficulties with the current classification system is maintaining objectivity in the face of biological distinctions which oversimplify our view of herpesviruses. These distinctions largely originate as a function of our inability to study the effects of herpesviruses in vivo, together with a limited availability of suitable culture systems for manipulating and studying these viruses in vitro. Biological properties that were intended to classify

herpesviruses in a scientifically-beneficial manner are wrought with a number of inconsistencies and paradoxes. Many of these have only recently come to light.

2. Epitheliotropism.

Epithelial cells are a critically important tissue for any herpesvirus replication strategy. However, relatively little is known about factors responsible for limiting or promoting growth in these cells. Despite all the emphasis on EBV's lymphotropic association with B-cells in Burkitt's lymphoma, the predominant malignancy associated with EBV is actually an epithelial malignancy, nasopharyngeal carcinoma. Although EBV and MDV are commonly labeled as 'lymphotropic' herpesviruses, oropharyngeal epithelial- and feather follicle epithelial cells, respectively, are responsible for the production and horizontal transmission of fully enveloped infectious virions (Calnek et al., 1970; Nazerian and Witter, 1970; Sixbey et al., 1984). The relatively scarce molecular details about MDV and EBV growth in these cells is largely a reflection of technical limitations which have traditionally hindered in vitro studies in epithelial cells.

3. Lymphotropism.

Although alpha- and betaherpesviruses are generally not referred to as 'lymphotropic herpesviruses' (in contrast to the gammaherpesviruses), lymphotropism is probably common to all herpesviruses. Its clinical significance has been cited for betaherpesvirus- (e.g. HCMV; Saltzman et al., 1988) and alphaherpesvirus infections, including those of HSV (Nahmias and Roizman, 1973), VZV (Grose, 1982), equine herpesvirus (EHV; Bryans, 1969; Scott et al., 1983), bovine herpesvirus (BHV; Nyaga and McKercher, 1980), pseudorabies virus (PRV; Wang et al., 1988; Wittmann and Rziha, 1989) and feline herpesvirus (FHV; Tham and Studdert, 1987).

Lymphotropism among alphaherpesviruses is often overlooked, despite its importance to pathogenesis and mortality. Upon further comparison with other alphaherpesviruses, a number of striking parallels are noted in comparison to MDV infections. With respect to T cell tropism, MDV and HSV are similar; replication of each is restricted to activated, Ia-bearing T cells (Braun et al., 1984; Calnek, 1986). In the case of MDV, these represent the target cells for transformation (see II.G.). HSV-2- and VZV infections, involving neonatal and immunocompromised hosts often result in severe systemic infections, characterized by widespread blood-borne dissemination of virus to multiple organs, often resulting in death (Grose, 1982; Nahmias and Roizman, 1973). Such infections bear a remarkable resemblance to the early events associated with MD pathogenesis. Both involve biphasic patterns of virus replication in lymphoid cells characterized by the development of a primary viremia which serves to disseminate virus to various organs. What follows is a brief period in which virus temporarily disappears from the blood in conjunction with the beginning of virus replication in various organs. Often a massive, secondary viremia follows and multiple lesions involving many organs are observed, ultimately leading to death. In both cases, overall viremia levels appear to directly correlate with death. The consequences of blood-borne MDV infections, particularly in young, genetically susceptible, antibody-free chickens often result in an early mortality syndrome leading to death, usually between 1.5 and 3 weeks post-inoculation (Witter et al., 1980). In such cases, tumor formation is absent; principal lesions are severe atrophy of the bursa and thymus, destruction of lymphoid cells, encephalitis, and an occasional focal necrosis of spleen and liver. As is the case with MDV, control of these blood-borne infections is largely influenced by immunocompetence and age (Calnek and Witter, 1991; Nahmias and Roizman, 1973). It will be interesting to see

whether the comparatively looser restrictions against severe pathogenesis by MDV in older birds (compared to HSV/VZV) are determined by intrinsic genetic properties of MDV or alternatively reflect fundamental differences associated with these immune systems.

4. Latency and neurotropism.

It is becoming increasingly more difficult to define persistent virus infections as strictly 'latent' or 'productive'. Such infections can either be productive or latent depending not only on the cell type infected, but on the 'activation' state of the cell (Ahmed and Stevens, 1990). Increasing lines of evidence indicate that generalizations regarding sites for virus persistence may be premature. There are indications that herpesviruses from all three subfamilies (e.g. EBV, CMV, PRV) may latently persist in *both* lymphoid *and* epithelial cells (Schrier et al., 1985; Stevens, 1989; Wittmann and Rziha, 1989). Recent evidence indicates that the alphaherpesvirus, EHV-1 can establish latent infections in T-lymphocytes (Welch et al., 1992), lending support to an earlier proposal characterizing EHV-1 as a T-lymphotropic herpesvirus (Scott et al., 1983).

Although alphaherpesviruses, are often distinguished by their ability to undergo latent infections in neural tissues, recent evidence suggests that in contrast to HSV, VZV establishes latent infections in satellite and perhaps other nonneuronal cells (Croen et al., 1988). The 'lymphotropic' MDV also appears to go latent in neural tissues and like VZV this appears to involve some of the same nonneuronal tissues, namely the nonmyelinated Schwann cells and satellite cells (Pepose et al., 1981). VZV's pattern of gene expression in nonneuronal nervous tissues appears to be entirely distinct from that observed in neurons latently infected by HSV (Croen et al., 1988). Together, the above findings suggest that latency may take on many forms; its nature reflecting the

different requirements and controls which limit productive or semi-productive infections, depending on the given cell type involved.

5. Further biological parallels: pathogenic manifestations attributed to MDV and alphaherpesviruses.

The 'neurotropic alphaherpesviruses' are characterized by a range of ocular and neurological manifestations. For example, HSV-1 is known to be a leading infectious cause of blindness and encephalitis. Despite all the attention emphasizing MDV's lymphotropic/oncogenic properties, MD is often regarded more as a neuropathological disease. Classical MD (also called fowl paralysis) is characterized by neural lesions resulting in peripheral nerve demyelination. Certain pathogenic MDV isolates are particularly distinguished by a marked propensity to cause reversible encephalitis (e.g. transient paralysis; Kenzy et al., 1973) or blindness (Ficken et al., 1991). While HSV-induced encephalitis is often mediated by a neurogenic pattern of infection, hematogenous spread has been clearly established, particularly with regard to HSV-2 infections involving neonatal and immunocompromised individuals (Nahmias and Roizman, 1973). The previously described association between VZV infections and the Landry-Guillain-Barre syndrome (Sanders et al., 1987) is of interest in light of the fact that MDV has been considered a useful animal model system for this neurological condition (Pepose et al., 1981; Hughes, 1990). Finally, accumulating evidence has established a common etiological link involving both MDV and HSV in the pathogenesis of atherosclerosis (reviewed by Fabricant, 1985; Hajjar, 1991).

F. Future trends in herpesvirus classification.

With the onset of recombinant DNA approaches to rapidly analyze genome structure and genetic organization (Buckmaster et al., 1988; Davison, 1992; Efstathiou et al., 1990; Gompels et al., 1988; Lawrence et al., 1990),

coupled with ever-expanding databases containing a wide range of nucleic- and predicted aa sequences representing all three herpesvirus subfamilies, a phylogenetically-based classification approach appears much more feasible at this time. Such a change would be desirable not only because of the problems and discrepancies associated with a biologically-based classification system, but because of the fact that phylogenetic relationships provide the most useful tools for addressing the genetic nature of phenotypic differences. A move towards a phylogenetically-based classification system would not require reclassification of most herpesviruses; it would simply place genetic relatedness as the dominant criteria for classifying herpesviruses.

IV. Evolution of herpesviruses.

A. Introduction: Biological observations and hypotheses.

It has often been said that the evolution of viruses is the evolution of their hosts. Since herpesviruses are characterized by the presence of an envelope acquired by budding from nuclear and/or Golgi body membranes, bacterial hosts appear unlikely to have played a role in the evolution of herpesviruses. Nevertheless, the widespread occurrence of herpesviruses in many varieties of eukaryotes is suggestive of a long evolutionary history. To gain further insight about a putative progenitor herpesvirus, Nahmias (1972) has suggested a concerted attempt to identify herpesvirus in primitive eukaryotic life forms.

A major theoretical challenge is understanding the balance between intrinsic virally-encoded (pathogenic) influences and those attributable to an immune system not adapted for dealing with such ordinarily harmless pathogens. To better appreciate this, it is necessary to consider some of the peculiarities of herpesviruses. In most cases, herpesviruses are relatively harmless in their natural host (beyond the newborn age). However, natural

transmission across normal host-species barriers can have devastating consequences. This is best exemplified by B virus and herpesvirus saimiri, two simian herpesviruses common to old- and new world monkeys, respectively. The former is almost invariably fatal to humans and various experimentally-infected hosts; the latter is harmless to squirrel monkeys, but highly oncogenic and fatal to owl monkeys (Barahona et al., 1974). In fact, isolation of herpesviruses from fatally-infected animals beyond the newborn age is generally an indication that another animal species was the source of the virus (Nahmias, 1972). Together with a long-lasting latent association with their host, these peculiarities suggest that herpesviruses have co-evolved with their host so as to cause little disturbance. According to the Theobald Smith doctrine (1934), these characteristics indicate that herpesviruses are ancient, well-adapted parasites (Wildy, 1973). By preserving the host, while at the same time allowing the survival of the virus and its ultimate dissemination to further hosts, often many years later, herpesviruses have an important edge over many other virus groups.

To explain the nature of this phenomenon, Nahmias (1972) has postulated the existence of a host factor 'X' which, when present, keeps the virus from harming its host. Because of the severity of infections in newborn- or immunocompromised individuals (non-human species as well), Nahmias has suggested that the 'X factor' is probably associated with the host's immune system. Such a proposal appears consistent with recent MDV studies suggesting that latency is maintained by the presence of a so-called latency-maintenance factor (LMF) present in conditioned media (Buscaglia and Calnek, 1988), which is known to be rich in cytokines, including the lymphokines interleukin-2 and gamma-interferon.

B. Observations and hypotheses from genetic studies.

1. Genetic relationships among herpesviruses.

i. Basis for genome structure diversity.

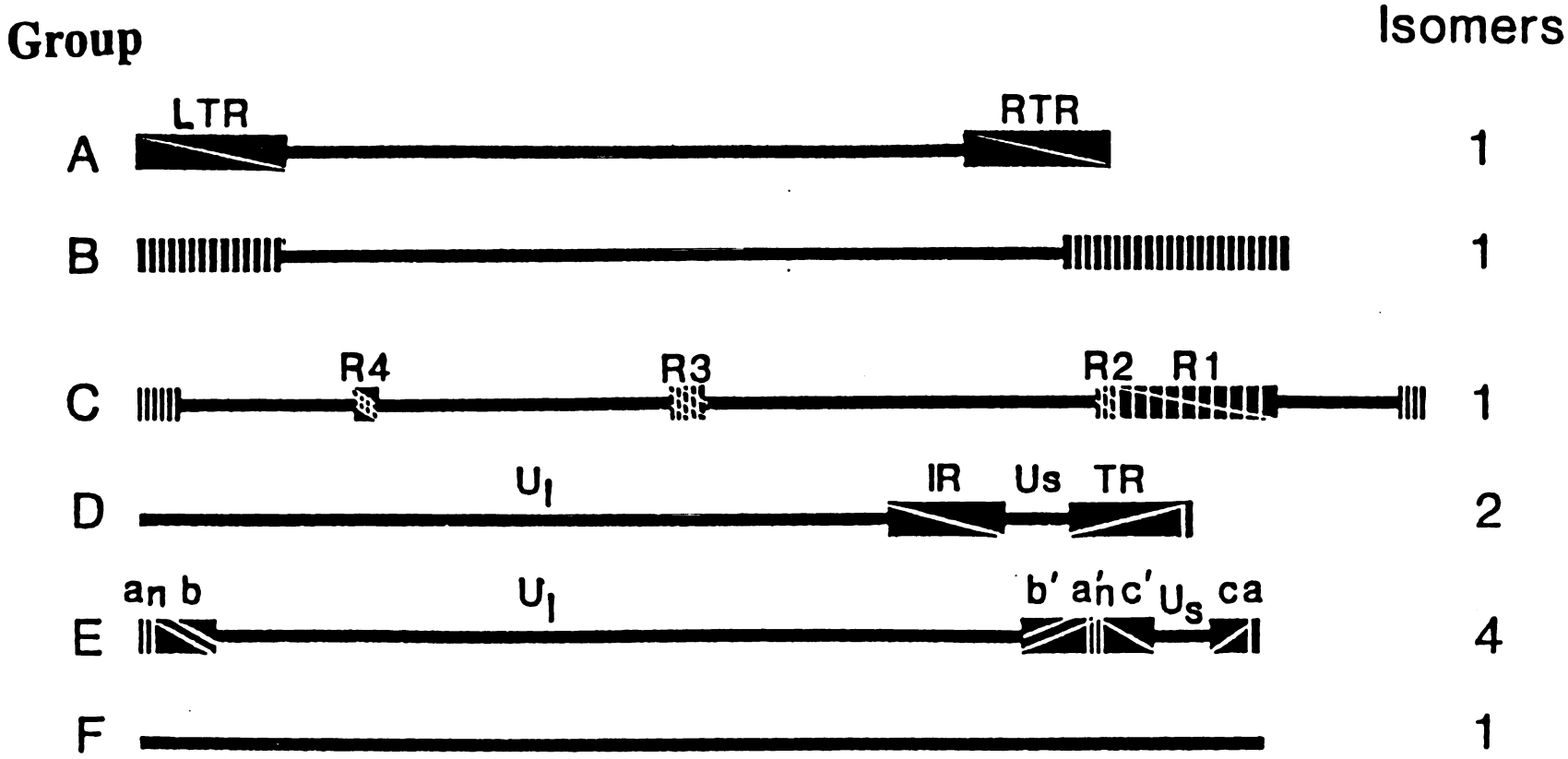
Herpesvirus genomes commonly contain a unique long region associated with various types of direct, inverted and/or internal repeat structures. These repeat structures vary in number, arrangement, and composition. Figure 2 illustrates five of the genome structures that have been presently identified (Roizman, 1990b). Genome structures do not necessarily reflect phylogenetic relationships. HSV and HCMV each have group E genome structures that can similarly isomerize. Moreover, HCMV also contain functionally homologous *a* sequences that can substitute as a cleavage/packaging signal for HSV-1 (Spaete and Mocarski, 1985). Conversely, closely-related viruses, such as PRV and HSV-1, differ in their genome structures more than distantly-related viruses, HCMV and HSV-1 (Figure 2).

Differences in organization of herpesvirus repeat regions are thought to be a reflection of the replication process, which appears to be intimately associated with the propensity to undergo recombination (Honess, 1984; Weber et al., 1988; Dutch et al., 1992). One consequence of the herpesvirus replication process is that internally reiterated sequences can mediate reciprocol recombination with their homologous repeat sequences elsewhere, resulting in the production of distinct isomers with unique regions inverted relative to one another. Located in the inverted- and terminal repeats of HSV, VZV, and HCMV are the so-called *a* sequences, which contain *cis*-acting signals for cleavage of viral concatamers and packaging of their unit-length genomes (Mocarski and Roizman, 1982; Spaete and Mocarski, 1985; Varmuza and Smiley, 1985). Originally it was suggested that *a* sequences mediate the inversion process; however, recent studies indicate that HSV-1 inversion/isomerization can be

FIGURE 2. Sequence arrangements in the 6 groups of herpesvirus genomes.

The genomes A, B, C, D, E, and F are exemplified by the channel catfish (CCV), herpesvirus saimiri (HVS), Epstein-Barr virus (EBV), varicella zoster (VZV), herpes simplex 1 (HSV-1), and tree shrew herpesvirus (TSHV). In the schematic diagram the horizontal lines represent unique or quasi-unique regions. The reiterated domains are shown as rectangles and are designated as left and right terminal repeats (LTR) and (RTR) for group A, repeats R1 to R4 for internal repeats of group C, and internal and terminal (IR and TR) repeats of group D. The termni of group E (e.g. HSV) consist of two elements. One terminus contains *n* copies of sequence *a* next to a larger sequence designated as *b*. The other terminus has one directly repeated *a* sequence designated as *c*. The terminal *ab* and *ca* sequences are inserted in an inverted orientation (denoted by primes), separating the unique sequences into long (U_L) and short (U_S) domains. Terminal reiterations in the genomes of group F have not been described. In group B, the terminal sequences are reiterated numerous times at both termini. The number of reiterations at each terminus may vary. The components of the genomes in groups D and E invert. In group D, the short component inverts relative to the long. Although the long component may also invert (rarely), most of the DNA forms two populations differing in the orientation of the short component. In the In the group E genomes, both the short and long components can invert and viral DNA consists of 4 equimolar isomers. From Roizman, 1990b.

SEQUENCE ARRANGEMENTS IN THE 6 GROUPS OF HERPESVIRUS GENOMES



mediated by repeat sequences idependant of the *a* sequences (Weber, 1988). The repeat sequences themselves appear to be inherently recombinogenic (Honess, 1984; Weber et al., 1990); this property applies to the *a*-sequence structure itself, apparently accounting for its own ability to undergo recombination and expansion (Umene, 1991). Recent evidence has shown that *a* sequences promote isomerization twice as readily as unrelated sequences, similar in size. More importantly, recombination between *a* sequences was found to occur at approximately the same time as replication (Dutch et al., 1992), lending further support to the idea that replication and recombination are closely linked (Honess, 1984; Weber et al., 1988; Weber et al., 1990). Together these features characterize the nature of herpesvirus replication-associated recombination, which is likely responsible for much of the structural- and genetic organizational divergence which characterize members of the herpesvirus family.

These aspects are particularly highlighted by the identification of PRV- and HCMV variants with extra pairs of inverted repeats which facilitate the generation of four- (Lomniczi et al., 1987) or eight genome isomers (Takekoshi et al., 1987). The four-isomer PRV group E (also called class E, type E or class 3) structures have been postulated to arise from a double-crossover event between two inversely oriented concatameric group D (also called class D, type D, or class 2) molecules (Lu et al., 1989). Under certain growth conditions PRV variants evolve to acquire group E structures which confer a selective growth advantage in some types of cells, but a selective disadvantage in others (Lomniczi et al., 1987; Reilly et al., 1991). These mutants were found to have acquired alternate cleavage/encapsidation sites by the juxtaposition of terminally-located sequences next to the internal inverted repeat (Rall et al., 1991); their subsequent cleavage has been directly linked to the inversion of the

L component (Kupershmidt et al., 1992). Based on additional factors, these results have been interpreted to suggest that the emergence of viral populations with group E genomes represents an adaptation to the prevailing conditions in a given host or target tissue (Rall et al., 1991).

An analogous process was postulated to account for the evolutionary divergence of HSV and VZV S regions from a common progenitor (Whitton and Clements, 1984a; Davison and McGeoch, 1986). Such a process is thought to have involved a series of homologous, semi-homologous and/or non-recipricol cross-over events that can potentially lead to the: (i) loss, gain, and/or diploidization of sequences; (ii) creation of new open reading frames (ORFs) and; (iii) recruitment (and substitution) of promoter and/or regulatory elements. This provides rationale for the observation that VZV lacks six HSV-1 U_S region homologs (US2, US4, US5, US6, US11, US12) and has two others (US1 and US10) diploidized and relocalized to the adjoining repeat regions (Davison and McGeoch, 1986). Support for this proposal has been gained from HSV-1 and -2 studies demonstrating that expansion and contraction of IR_S/TR_S- and U_S regions can indeed occur (Brown and Harland, 1987; Umene, 1986).

An additional level of diversity may have been introduced by the existence of an error-prone DNA polymerase. Previous studies have suggested that wild-type HSV strains have such an enzyme (Hall et al., 1985); analogous results have been interpreted as accounting for the extensive divergence of retroviruses, such as HIV. To account for the wide variations in G+C content (even among closely-related members), non-selective forces resulting from intrinsic mutational biases in the DNA replication machinery have been postulated (Honess, 1984), aided by the help of recombination (McGeoch, 1987-suppl). Further changes may have occurred as a consequence of different viral enzymes involved in nucleotide metabolism (Honess, 1984).

ii. Convergent vs. divergent evolution.

At present, HSV-1 (McGeoch et al., 1988), VZV (Davison and Scott, 1986), HCMV (Chee et al., 1990b), and EBV (Baer et al., 1984) have been completely sequenced. Compared to other viruses, their genomes are complex, ranging between 124,884- (VZV) and 229,354-bp (HCMV) in length and containing 70- (VZV) to 200- (HCMV) genes. Together they represent at least one member of each of the three herpesvirus subfamilies. These four herpesviruses represent at least one member for each of the three herpesvirus subfamilies. Because of their clinical significance, more is known about the nature of human herpesvirus genes and their gene products in comparison to those of animal systems. In conjunction with this information, these sequence databases provide an especially important foundation for phylogenetic- and molecular studies involving less characterized non-human systems.

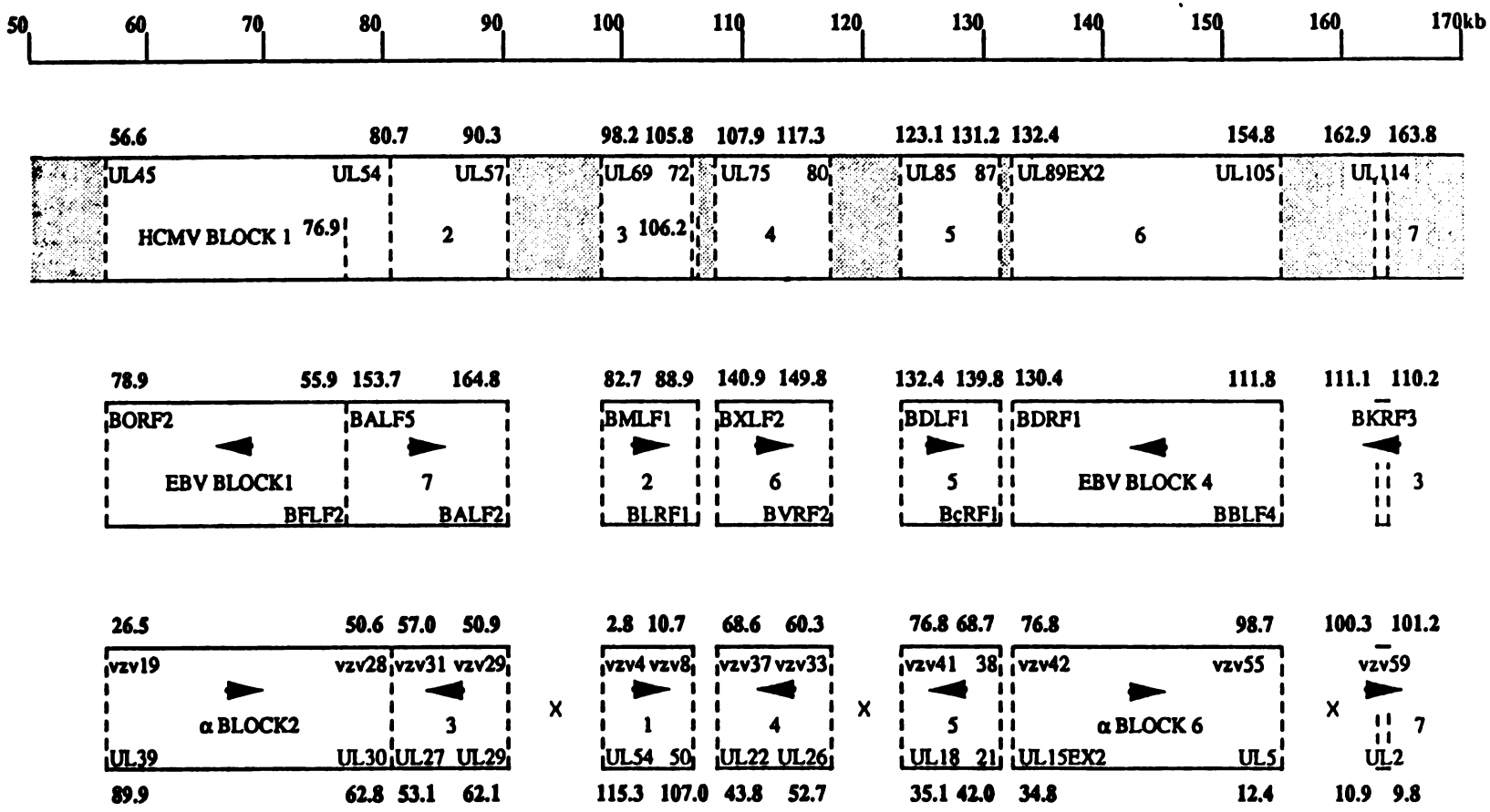
Homology comparisons between these genomes indicate that members of all three lineages share a common 'core' of about 30 related genes located in the unique long regions of their respective genomes (Chee, 1990b). The presence of these common genes suggests that members of the three herpesvirus subfamilies have all evolved from a single progenitor by a process referred to as *divergent evolution.* Positional localization of these core genes in the alpha-, beta-, and gammaherpesvirus lineages suggests that such an evolutionary process would have required large-scale U_L region rearrangements (Davison and Taylor, 1987; Chee et al., 1990b; Kouzarides et al., 1987). Figure 3 depicts the rearrangement and organization of homologous gene blocks in the genomes of HCMV, EBV, VZV, and HSV-1. The gross colinearity of U_L gene organization between VZV and HSV-1 is reflected in the fact that their 7 gene blocks are identically oriented.

FIGURE 3. Conserved blocks of sequence between HCMV, EBV, VZV, and HSV-1.

The *uppermost map* represents a section of the HCMV UL inicated by the scale at the top of the diagram. The *middle map* depicts regions of EBV conserved with HCMV, and the *lower map* shows VZV (above) and HSV-1 (below) homologies, also to HCMV. Only the HCMV map is drawn to scale. All homologies found so far with the alpha- and gamma-herpesviruses are located within the *unshaded* sections of the HCMV UL. The approximate boundary positions of the homology blocks within their respective genomes are marked in *boldtype* in kilobase pairs (positions are taken from Chee et al., 1990b; Baer et al., 1984; Davison and Scott, 1986; McGeoch et al., 1988). Note that these numbers represent only the termini of the endmost detected homologous frames in each genome, and that some of these homologies are tentative (Table 2). The names of the frames are given. The orientation of each of the blocks in EBV and VZV (but not HSV-1) is shown relative to their published maps (Baer et al., 1984; Davison and Scott, 1986); *rightward arrowheads* denote collinearity. The order of the blocks within each genome is shown by a *block number*; these read from left to right across the genome in ascending order. Three of the five locations of nonhomologous reading frames found between the UL regions of HSV-1 and VZV are marked in the lower map (*x*) (McGeoch et al., 1988).

From Chee et al., 1990b.

CONSERVED BLOCKS OF SEQUENCE BETWEEN HCMV, EBV, VZV, AND HSV-1



An alternative, non-mutually-exclusive possibility for herpesvirus evolution is *convergent evolution.* This form of evolution would predict that the same mechanism from which herpesviruses originally evolved would have independently repeated itself. The recent sequence analysis of CCV (see III.D.3.) highlight genetic properties which are entirely distinct from the alpha-, beta-, and gammaherpesvirus lineages. Such findings provide support for the view that herpesviruses have evolved both divergently *and* convergently.

iii. Differences characterizing the three phylogenetic lineages.

Aside from the 'core' of genes conserved by all herpesviruses (except CCV), each lineage contains a unique set of genes which are specific to its phylogenetically-related members. Some of these appear to have been transduced from cellular genes (Sugden, 1991). For example, the EBV BCRF-1 gene encodes a homolog of interleukin-10. Gammaherpesviruses, appear to have evolved their own characteristic set of unique genes important for maintaining latent infections, as well as specifying functions responsible for immortalization and/or transformation (Kieff and Liebowitz, 1990). Despite their structural differences, EBV and HVS encode serologically-related proteins and share a common organization of coding sequences which differs from that of the alpha- and betaherpesviruses (Davison and Taylor, 1987; Efstathiou et al., 1990; Gompels et al., 1988; Nicholas et al., 1992b). However, recent studies indicate that these two gammaherpesviruses significantly diverge in at least two locations (Nicholas et al., 1992b). This includes the absence of two blocks of genes in HVS which correspond to EBV genes (encoding EBNAs) and cis-acting signals (oriP) associated with latent growth, as well as those which disrupt latency and promote lytic growth (e.g. BZLF1). Although the nature of genes responsible for latency in HVS are not yet characterized, HVS has incorporated its own unique

repertoire of genes, including some which bear a close resemblance to members of the D-type cyclin- and G-protein-coupled receptor (GCR) family of proteins (Nicholas et al., 1992a).

With a 229,354 bp genome, the betaherpesvirus, HCMV is much larger than the genomes of alpha- and gammaherpesviruses. As such, it has incorporated a more diverse set of genes, a number of which bear cellular counterparts. This includes an MHC class-I-related gene (Beck and Barrell, 1988) involved in preventing immune surveillance (Browne et al., 1990) and a family of 3 GCR-related genes (UL33, US27, and US28; Chee et al., 1990a) related to recently described GCR homologs in HHV-6 (Neipel et al., 1991) and HVS (Nicholas et al., 1992a). The U_S localization of US27 and -28 may appear to suggest host-derived acquisition independent from those of HHV-6 and HVS. On the other hand, a common U_L localization for the HHV-6 GCR homolog (ORF3) and HCMV UL33 suggests a common phylogenetic origin, possibly distinct from US27 and -28. The latter are tandemly arranged, presumably evolved by duplication and divergence.

The HCMV GCR-related gene family represents one of at least nine different sets of homologous gene families, the majority of which are located in the U_S region of HCMV (Weston and Barrell, 1986; Chee et al., 1990b). These account for at least 21 genes, each distinguished by a unique aa motif pattern characteristic for each family (Chee et al., 1990b). Members of the US6 glycoprotein family (US6, -7, -8, -9, -10, and -11) exemplify recent findings indicating that HCMV's U_S region specifies an important locus for genes that are nonessential for growth in cell culture (Jones et al., 1991; Jones and Muzithras, 1992; Kolbert-Jons et al., 1991).

HCMV and HSV-1 both contain invertible S components with genes that are unique to alpha- and betaherpesviruses; however, aside from their basic

structural similarity, these two regions bear no sequence homology with one another. Nevertheless, these two regions exhibit remarkable parallels in their nature. Each encodes a set of genes unique to members of their subfamily. Like HCMV, alphaherpesviruses contain a similar cluster of U_S region genes that are nonessential for growth in cell culture (de Wind et al., 1990; Longnecker and Roizman, 1987; Longnecker et al., 1987; Weber et al., 1987). These appear to encode *supplementary essential* functions that have presumably evolved to facilitate the efficient dissemination of virus in their host tissues (Roizman, 1990a; further discussed in V.B.). Furthermore, like HCMV, alphaherpesviruses, such as HSV-1, contain a set of tandemly arranged glycoprotein genes which appear to have similarly evolved by a process of gene duplication and divergence (McGeoch, 1990). It will be interesting to see whether other betaherpesvirus S regions exhibit a similar genetic divergence which characterizes alphaherpesviruses (Brunovskis and Velicer, 1992; Davison and Wilkie, 1983; Davison and Scott, 1986; further discussed in V. A.).

Although the acquisition of a progenitor S region appears to have been an event coinciding with establishment of the alphaherpesvirus lineage (Davison and McGeoch, 1986; Davison and Scott, 1986; Davison and Taylor, 1987; McGeoch, 1990), this may not be the case with betaherpesviruses. Despite a closer phylogenetic origin between HCMV and HHV-6 (Lawrence et al., 1990; Neipel et al., 1991), the latter contains a smaller (appr. 162-168 kb) group A genome structure (Figure 2) lacking an S component (Lindquester and Pellett, 1991; Martin et al., 1991). HCMV may have acquired an S component relatively recently in its evolution from a progenitor betaherpesvirus. Alternatively, HHV-6 may have lost its S component, perhaps reflecting a move towards a more lymphotropic existence.

2. Latency.

i. Introduction: EBV and HSV.

In recent years, numerous laboratories have sought to identify viral products which maintain the latent state. In the EBV system, such studies have identified a complex transcriptional pattern responsible for the expression of a small subset of EBV-encoded gene products in latently infected B-cells. Interestingly, the particular pattern of expression can vary between different B-cell subtypes EBV-transformed epithelial cells (Klein, 1989). While some of the functions expressed relate to immortalization and/or transformation, one unifying feature characterizing persistence in these different infections is the consistent identification of EBNA-1, a trans-acting factor necessary for replication and maintenance of episomal EBV genomes. Recent data suggest that such results may be flawed by the artifactual nature of the in vitro culture systems employed raising important concerns about the relevance of EBNAs to normal latent infections. A recent PCR-based study (Qu and Rowe, 1992) using uncultured peripheral blood lymphocytes from healthy, latently-infected individuals has found, contrary to expectations, an apparent dispensability for EBNA expression, instead implicating a central role for the TP membrane protein, previously identified in 'latently' infected tissue culture cells (Kieff and Liebowitz, 1990).

HSV-1 studies have thus far failed to identify a single viral gene actively involved in neuronal latency. In spite of the initial enthusiasm concerning identification of non-polyadenylated transcripts expressed in latently-infected cells (e.g. latency-associated transcripts, LATs), LAT mutants are nevertheless able to facilitate the establishment and reactivation of latent infections (Steiner et al., 1989). These findings suggest that, in some cases, latency may be more a function of the host limiting the expression of viral genes important in productive

infection either by repressing their expression (Lillycrop et al., 1991) or failing to express critical transcription factors necessary for their activation (Garcia-Blanco and Cullen, 1991).

ii. CpG content.

In actively dividing cells, CpG dinucleotides of vertebrate genomes are susceptible to methylation. This leads to the production of 5-methylcytosine, which is deaminated at high frequency to form thymine. As a consequence of this propensity for methylation, all vertebrate genomes thus far analyzed have disproportionately *low* CpG frequencies and disproportionately *high* frequencies of TpG dinucleotides compared to those predicted on the basis of mononucleotide compositions (Bird, 1980). Herpesviruses lack methylation system of their own, yet recent studies have illustrated a striking pattern of CpG frequencies largely consistent with the current biologically-based classification system (Honess et al., 1989). Gammaherpesviruses, such as EBV and HVS are characterized by significant CpG deficits associated with corresponding excesses of TpG. In contrast, alphaherpesviruses, such as HSV, VZV, and PRV fail to exhibit discrepancies in their dinucleotide frequencies, while the betaherpesvirus, HCMV, has properties intermediate between those of the other two families. Local CpG deficits and TpG excesses were resticted to the immediate early genes of HCMV. The latter result, while difficult to interpret, reflects the biological ambiguities associated with this unusual group of herpesviruses. The CpG deficiencies common to all gammaherpesviruses thus far analyzed (EBV, HVS, and MHV-68; Efstathiou et al., 1990; Honess et al., 1989), have been interpreted as a consequence of their long-term maintenance in actively-dividing lymphocytes subject to methylation, in contrast to alphaherpesviruses, which are latently-maintained in a non-dividing methylation-free environment.

Consistent with their close phylogenetic relationship with alphaherpesviruses, MDV has not been found to exhibit CpG deficiencies (Honess et al., 1989; Ross et al., 1991; Brunovskis and Velicer, 1992a). On the surface, this would appear to conflict with the fact that MDV can go latent in lymphocytes. The lack of CpG deficiencies for MDV have been interpreted to suggest that the epidemiologically significant form of MDV transmitted in nature is unlikely to be one whose precursor derives from latently-infected T-lymphocytes (Honess et al., 1989). Although MDV has been shown to be methylated in lymphoid tumor cells (Kanamori et al., 1987), these findings are not irreconcilable with such predictions.

It is worth questioning whether MDV persistence and shedding are actually derived from 'latently'-infected lymphocytes. There is surprisingly little known about the long-term maintenance of MDV. Only one study has been conducted that would support the notion of long-term MDV latency in lymphocytes (Witter et al., 1971). Unlike classic co-cultivation assays for latency, virus could only be rescued from a small proportion of convalescent birds 76 weeks postinfection. This raises a number of questions. Does MDV employ an active, genetically-determined strategy for maintaining long-term latency in lymphocytes (like EBV)? Could the difficult-to-detect viremia possibly reflect persistence in FFE cells from which periodic bouts of productive replication serve to non-productively re-infect lymphocytes in a chronic fashion? It should be noted that, despite their ability to protect against T-cell-induced lymphomas, vaccinated birds still manage to shed superinfected MDV (Purchase and Okazaki, 1971). Does this reflect persistence in immunologically-protected FFE cells?

As described in II.D., MDV is extremely efficient in horizontal transmission. Unlike other herpesviruses, which are usually transmitted during

episodes of acute infection, MDV is released in a form that can remain infectious for long periods of time (>200 days) at ambient room temperatures (Carozza et al., 1973). Residual MDV associated with keratinized material near the feathers has been previously suggested to contribute to the long-term shedding of infectious virus (Johnson et al., 1975). MDV's ability to initiate a fully productive infection is intimately associated with the state of FFE cell differentiation. Nonproductive infections involving the basal layers of FFE cells can eventually proceed to a productive infection upon differentiation (Johnson et al., 1975). It may be worth considering whether MDV can maintain itself in a non-productive state in basal layers of the FFE from which it ultimately proceed to a productive infection (and shedding) upon differentiation. The latter could also account for chronic, non-productive lymphocyte reinfections, as well as reinfections of new basal layers in order to maintain MDV's persistence for subsequent periodic sheddings. A similar idea has been proposed to emphasize a more prominent role for oropharyngeal cells in EBV persistence (Allday and Crawford, 1988; Rickinson et al., 1985). However, consistent with predictions based on CpG deficiencies, recent findings appear to rule out such a hypothesis for EBV (Gratama et al., 1988; Niedobitek et al., 1991). Finally, with regard to MDV, it may be worth adding that a chicken succumbing to a tumor following a 'latent' lymphocyte infection would be unable to subsequently transmit virus if it were dead!

iii. Codon usage.

Despite new questions concerning the nature of genes expressed in cells latently infected with EBV, Karlin and colleagues (1990) have recently identified a potentially important contrast in codon usage between genes thought to be specific for productive- or latent infections. In particular, they found a statistically significant decrease (20%) in the percentage

of G or C in codon site 3 (S3 percentage) for genes expressed in latent infections. This and other disparate features of codon usage were interpreted as reflecting an adaptation to minimize the deleterious consequences to the host during latent infections.

3. Sequence analysis as a marker for past events.

If we assume that the evolution of herpesviruses is synonomous with the evolution of their hosts, then it follows that sequence comparsions between viral genes and their cellular counterparts offer a potential strategy which can lend insight into the past history of a given virus or its host. Phylogenetic trees have often been used to estimate the divergence time between host organisms and virus which infect them. Amino acid sequence comparisons between the thymidine kinase genes of HSV and marmoset herpesvirus (MHV) suggest that HSV-1 and -2 diverged from one another 8-10 million years ago (Gentry et al., 1988). This time span was consistent with other phylogenetic trees similarly constructed using herpesvirus sequences from three independent sets of related proteins. HSV-1 and -2 are primarily transmitted by oral and genital routes of infection, respectively. Considering the fact that the closely related B virus is transmitted by both routes, in addition to the above divergence time and a number of other factors, a provocative suggestion has emerged. By this, the 8-10 million year divergence time was predicted to result from changes in human sexual behavior influenced by (i) adoption of a generally upright position resulting from a change towards bipedalism; (ii) adoption of close face-to-face ventral-ventral mating, and (iii) an increased female sexual appetite, not limited by menstrual cycles (Gentry et al., 1988). These changes were considered necessary and appropriate for the microbiological isolation required to bring about the above divergence.

V. Characterization and significance of the alphaherpesvirus S region.

As previously noted (III.D.), alphaherpesvirus S regions (or S component) are covalently linked to an L region and consist of a unique short (U_S) segment bounded by a pair of inverted repeats (IR_S/TR_S, or simply R_S). The S regions of alpha- and betaherpesviruses bear no phylogenetic relationship to one another. The remainder of this review will summarize the significance and current state of knowledge concerning the various genes and products encoded by alphaherpesvirus S regions of human and animal systems. Figure 4 identifies alphaherpesvirus homologs which have been thus far identified. Names common to each system are listed below each box. Inasmuch as most S region homologs have HSV-1 counterparts, for which much of our current knowledge is derived, their identification will be simplified by the use of nomenclature emphasizing their homologies to HSV-1. Thus PRV gp50, a homolog of HSV gD (Figure 4) will be referred to as PRV gD or PRV US6. The term 'HSV' will be used in situations equally applicable to both HSV-1 and -2 (as before).

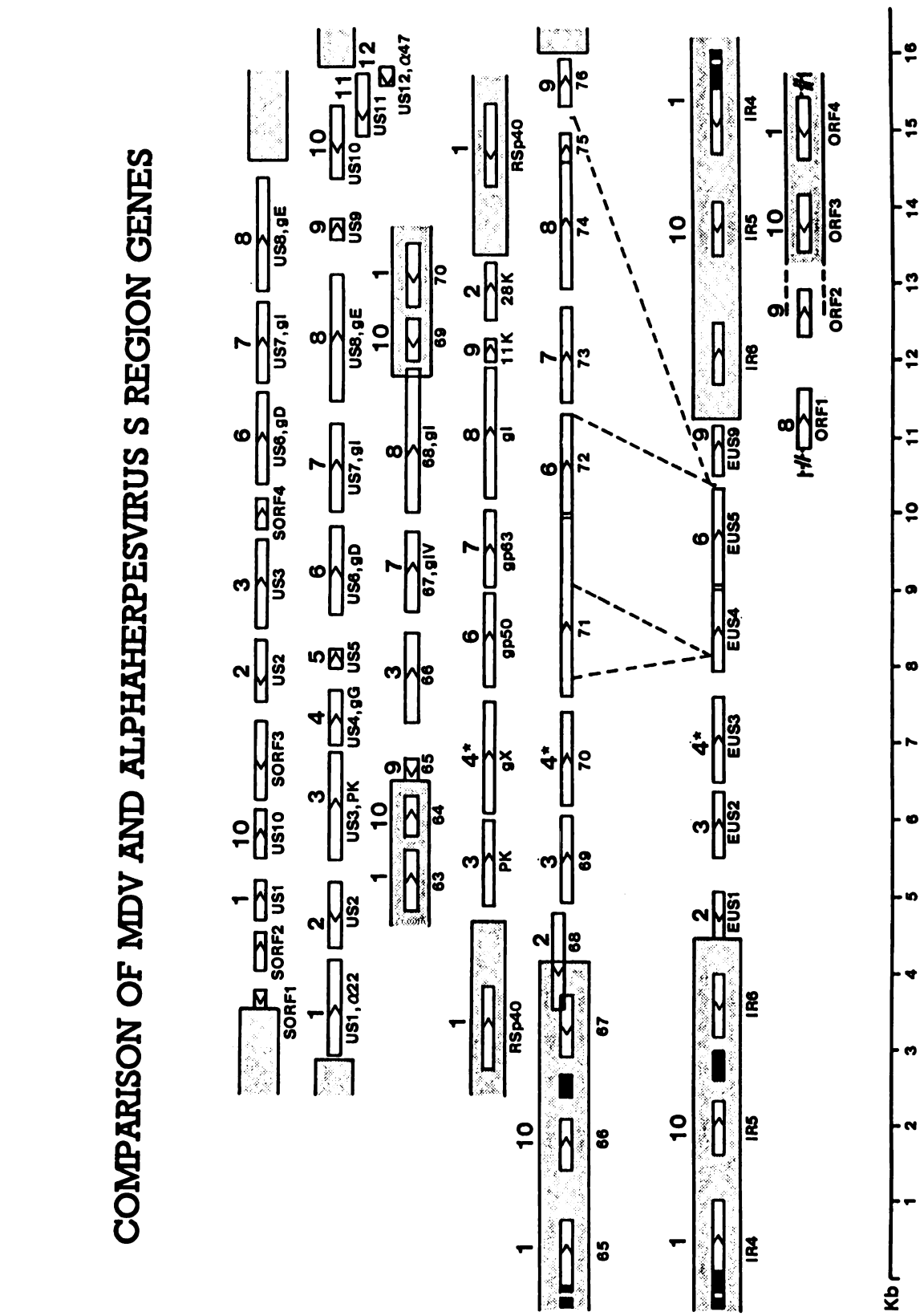
A. S region divergence.

Except for two HSV-1 U_L region genes (UL45, UL56), the remaining 54 all possess an equivalent in VZV (McGeoch et al., 1988). Unlike the L region, which shows a great deal of conservation, alphaherpesvirus S regions appear to be much more diverse. This was first observed in DNA-DNA hybridization studies comparing homologies between HSV-1, or HSV-2 and with EHV-1, PRV, and or VZV (Davison and Wilkie, 1983). Despite obvious homologies between their U_L regions in a colinear fashion, no homology could be detected between the U_S regions of HSV-1 and -2 and their alphaherpesvirus counterparts. Only after adjusting the formamide concentration to 30% and the hybridization temperature to 37 C, could a hybridization signal be observed (between HSV-1 and VZV; Davison and Wilkie, 1983). The overall divergence of

FIGURE 4. Comparison of MDV and alphaherpesvirus S region genes.

Based on published S region ORFs or unpublished ORFs accessible from GenBank (Audonnet et al., 1990; Breeden et al., 1992; Colle et al., 1992; Cullinane et al., 1988; Davison & Scott, 1986; Holden et al., 1992a, 1992b; McGeoch et al., 1985; Petrovskis et al., 1986a, 1986b; Petrovskis & Post, 1987; Telford et al., 1992; van Zijl et al., 1990; Zhang & Leader, 1990). Numbers above boxes refer to homologs based on relation to HSV-1 U_S ORF nomenclature (McGeoch et al., 1985). Polypeptide designations common to each system are listed below each of those boxes where applicable. Larger, stippled boxes refer to identified IR_S, TR_S, and/or R_S regions. Broken lines define regions deleted from the EHV-1 Kentucky A cell culture strain relative to that of the EHV-1 Ab4p field isolate strain. Bolded areas identify repeat sequences present in the two EHV-1 strains. Dashed lines extending from the stippled box of the EHV-4 TR_S region reflect uncertainty regarding the precise location of its U_S-TR_S junction (Cullinane et al., 1988). Asterisks refer to homologs which show relatedness to HSV-2 US4, rather than HSV-1 US4.

COMPARISON OF MDV AND ALPHAHERPESVIRUS S REGION GENES



U_S regions is further emphasized by the lack of hybridization between the U_S regions of closely-related viruses. Despite widespread hybridization between the genomes of EHV-1 and -3 (Baumann et al., 1986) and MDV and HVT (Igarashi et al., 1987), their corresponding U_S regions failed to exhibit detectable homology.

Sequence analysis has confirmed the diversity characterizing alphaherpesvirus S regions as their most divergent coding region (Brunovskis and Velicer, 1992a; Davison and McGeoch, 1986). Figure 4 illustrates the nature of this divergence. For example, the U_S region of HSV-1 is 13 kbp, compared to VZV's which is just over 5 kbp. VZV lacks six HSV-1-related homologs (US2, -4, -5, -6, -11, and -12; Davison and McGeoch, 1986); MDV lacks five such homologs (US4, -5, -9, -11, and -12), yet it contains at least four ORFs not common to alphaherpesviruses (Brunovskis and Velicer, 1992a). EHV-1 contains three such ORFs. The relative position of repeat junctions and homologs can vary greatly (Figure 4). Single-copy U_S homologs may be similarly- (e.g. US3, -4, -6, -7, and 8) or differently arranged (US2, -9) relative to one another. Some may be present either as single-copy U_S region genes or as two-copy repeat region genes (US1, -10). S region gene homologies are generally apparent only at the aa level. Although conserved amino acid regions are readily detectable, they are often restricted to particular locations. Such homologs often exhibit significant differences in length, transcriptional characteristics, genomic localization, and functional activity (Figure 4).

B. Presence and significance of supplementary essential genes.

To understand the nature of viral functions responsible for differences in pathologic- and biologic- properties it is necessary to identify and characterize the so-called 'nonessential', 'dispensable', or *supplementary essential* genes (for discussion, see Roizman, 1990a). In contrast to *minimally*

essential genes, which are necessary for replication, packaging and infection of cells in culture, the supplementary essential genes confer functions allowing for the efficient dissemination, growth, and maintenance in various tissues in the face of an immune system poised for its elimination. Achieving such goals has necessitated the development and evolution of elaborate, poorly understood mechanisms to cause immunosuppression, avoid immunosurveillance, and allow for the establishment and maintenance of a latent growth state and its subsequent reactivation to facilitate further dissemination to new hosts. Many of the distinct virus type-specific biological properties are likely to be determined by the supplementary essential genes, whose true function is probably only apparent *in vivo*. While these functions are likely to benefit the virus in furthering its existence, their presence is often associated with marked pathologic consequences to the host.

In recent years clusters of supplementary essential genes have been found in the S regions of HSV-1 and HCMV (Jones et al., 1991; Jones and Muzithras, 1992; Kolbert-Jons et al., 1991; Longnecker and Roizman, 1987; Longnecker et al., 1987; Weber et al., 1987). Such clustering can extend to other regions as well (Barker and Roizman, 1990; Baines and Roizman, 1991). Of the 12 HSV-1 U_S region genes, 11 have have been found to be dispensable for growth in cell culture (Longnecker et al., 1987). A viable PRV mutant has recently been created which lacks all four of the known nonessential PRV glycoprotein genes, including three U_S region genes (Mettenleiter et al., 1990b). Glycoprotein D (US6) is considered the exception to nonessential U_S genes. However, unlike HSV, PRV gD mutants can still grow in a cell-cell manner. (Peeters et al., 1992; Rauh and Mettenleiter, 1991). VZV lacks such a homolog altogether. Some 'dispensable' genes can become 'essential' when the function of another nonessential gene is lost. PRV gI (homologous to HSV gE) was

recently shown to be *essential* for growth in a gIII (homolog of HSV gC)-minus background (Zsak et al., 1992).

Unlike their wild-type parents, HSV U_S region gene mutants grow poorly in animal host models and are consistently associated with reduced levels of virulence and/or the capacity to induce latency (Meignier, 1988). Similarly, most (but not all) PRV U_S mutants are markedly attenuated and are less able to spread in both pigs and rats (Card et al., 1992; Kimman et al., 1992; Pol et al., 1991). The importance of 'nonessential' genes is highlighted by a failure to identify naturally occurring deletion mutants. MD vaccine studies have indicated that protection against tumor induction is largely effected by neutralizing the initial spread of primary infections. An understanding of mechanisms for dissemination, spread and characterization of tissue-specific factors that effect these processes are essential if we are to comprehend the pathologic- and biologic properties associated with herpesvirus infections.

Despite sharing similar aa sequences, herpesviruses exhibit strikingly different biologic- and pathologic expressions. This is not only true of closely related viruses such MDV and HVT, but is equally applicable comparing HSV-1 with -2 (Craig and Nahmias, 1973; Nahmias and Roizman, 1973). If we consider the proposal that the lymphotropic properties of MDV and HVT are unlikely to be determined by molecules homologous to those of EBV (Lawrence et al., 1990), questions arise concerning the nature of genes conferring this ability (as well as its inability to grow in neurons). It has been suggested that 'the delineation and evolutionary relatedness of genes responsible for biological properties may be a more significant criterion for both evolutionary relatedness and classification than the arrangement and evolution of genes conserved throughout the family Herpesviridae, although they are not yet known' (Roizman, 1990b). Although such a proposal is clearly debatable, the fact that U_S- and

other S region genes originate from an alphaherpesvirus-specific area exhibiting significant genetic diversity, pathogenic potential, and putative *supplementary essential* gene functions, together suggest the likelihood of such genes fitting the 'although they are not yet known' category above.

C. Regulation of herpesvirus gene expression: immediate-early genes.

All herpesviruses thus far analyzed undergo a temporal program of coordinated gene expression characterized by the initial expression of immediate-early (IE) (or alpha) gene products critical in triggering the subsequent expression of latter classes of viral products, whose synthesis may lack a dependence on viral DNA replication (early, E or beta) or require it (late, L or gamma) (Honess and Roizman, 1974). Activation of HSV-1 IE genes during productive infections is largely attributed to the activity of the U_L-encoded (UL48) gamma product, VP16 (also called Vmw65, a-TIF) which comes already packaged in the virion (Roizman and Sears, 1990). Because of their importance to the coordinate, sequential regulation of herpesvirus gene expression and the control of latency, products and homologs of ICP0, -4, -22, -27, -47 and VP16 have been the focus of numerous studies in recent years.

The five IE HSV-1 infected cell polypeptides (ICP) and their transcripts are listed in Figure 5 and Table 1. Nucleotide sequencing nomenclatures have alternatively referred to α27, α0, α4, α22, and α47 as UL54, IE110, IE175, US1, and US12, respectively (McGeoch et al., 1985; McGeoch et al., 1986; McGeoch et al., 1988; Perry and McGeoch, 1988). Similarly, their VZV counterparts (except α47 which is lacking) are represented by genes 4, 61, 62, and 63/70, respectively (Davison, 1986b). The U_L-encoded ICP27 homologs bear a distant relation with the EBV BMLF1 gene (Davison and Taylor, 1987), in contrast to the other HSV IE genes and their homologs, which appear unique to alphaherpesviruses.

FIGURE 6. Map of HSV immediate-early mRNAs.

A simplified map of the HSV genome and the positions and orientations of the immediate-early mRNAs. From Whitton and Clements, 1984b.

TABLE 1. The two parallel nomenclatures of the HSV IE gene products.

Shown are the sizes of the proteins as estimated by polyacrylamide gel electrophoresis in the presence of SDS, the sizes of the primary unmodified amino acid sequence deduced from the DNA sequence and the size (in nucleotides) of the corresponding mRNAs. From Everett, 1987.

MAP OF HSV IMMEDIATE-EARLY mRNAs

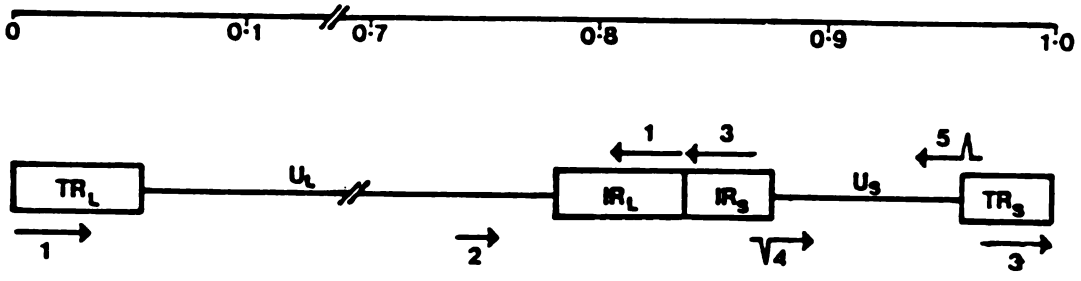


Table I. *The IE genes of HSV-1 and their products.*

Glasgow system		Chicago system		Size on SDS-PAGE	Size from sequence	Size of mRNA
Gene	Product	Gene	Product			
IE-1	Vmw110	α0	ICP0	110kd	78452	2684
IE-2	Vmw63	α27	ICP27	63kd	55376	1690
IE-3	Vmw175	α4	ICP4	175kd	132835	4259
IE-4	Vmw68	α22	ICP22	68kd	46521	1666
IE-5	Vmw12	α47	ICP47	12kd	9792	1772

Figure 6 shows the relative polarity and location of HSV-1 IE genes. It is important to emphasize that, except for ICP27, all of the IE products have promoters and/or products located in the repeat regions. Considering the enhanced mutability of the repeat regions, this observation underscores the potential for regulatory divergence among the various alphaherpesviruses (see below). Unlike ICP4 and ICP27, the other three IE products are produced from spliced transcripts. The IE-4 and IE-5 transcripts employ identical promoters located in the IR_S and TR_S regions (Figure 5). These spliced transcripts share a common non-coding exon located in the repeats which is spliced to unique ICP22- or ICP47-specific sequences located at either end of the U_S region (Watson et al., 1981; Rixon and Clements, 1982). In contrast to the unspliced ICP4 message of HSV-1, ICP4 transcripts of EHV-1 and BHV-1 are characterized by a complex pattern of splicing and regulation (Harty and O'Callaghan, 1991; Wirth et al., 1991; Wirth et al., 1992).

With the exception of ICP47 (Marsden et al., 1982), all of the HSV-1 IE products are phosphorylated and predominantly localized to the nucleus (for review of properties, see Everett, 1987). Most attention has been focused on ICP0, ICP4, ICP27, and their homologs, as they are known to be involved in the regulation of IE, E, and L gene expression. Despite impaired growth under certain circumstances (Post and Roizman, 1981; Longnecker and Roizman, 1986; Sacks and Schaffer, 1987) ICP0, ICP22, and ICP47 have been found to be dispensable for growth in cell culture, in contrast to the 'essential' genes which code for ICP4 and ICP27 (Roizman and Sears, 1990). Since ICP0 can trans-activate IE promoters (like VP16), it is thought to play an important role during the initial stage of reactivation which occurs in the absence of virion proteins such as VP16 (Cai and Schaffer, 1992). In this way ICP0 could be functionally analogous to the EBV ZEBRA product implicated as the key regulator

responsible for the shift between latency and lytic growth (Cai and Schaffer, 1992; Harris et al., 1989; Miller, 1990).

D. Characterization of S region genes.

1. IE175 (ICP4).

ICP4 is the only ORF located in the IR_S/TR_S regions of HSV (McGeoch et al., 1986) and is the major regulatory protein involved in activation of beta- and gamma genes, as well as repression of alpha genes, including itself (for review and references, see Papavassiliou et al., 1991). The HSV-1 N-terminal half apparently contains most of the functional domains responsible for nuclear localization, phosphorylation, DNA binding, transactivation, autoregulation (DeLuca and Schaffer, 1988); the role of the C-terminal half is more obscure. The nuclear-localized ICP4 product is found as a homodimeric complex (Metzler and Wilcox, 1985), with dimerization properties that map to its DNA-binding domain (Everett et al., 1991). Extensive posttranslational modifications, including phosphorylation, ADP-ribosylation, adenylation, and guanylation account for numerous bands on two-dimensional gels differing in apparent molecular weight and charge (Ackermann et al., 1984). Three phosphorylated forms (ICP4a, 4b, and 4c) have been recognized by one-dimensional SDS-PAGE (Wilcox et al., 1980; Ackermann et al., 1984). Phosphates have been reported to cycle on and off ICP4 (Wilcox et al., 1980) and to affect its interaction with infected- and uninfected cell factors in the activation of beta and gamma genes (Papavassiliou et al., 1991). Other features appear consistent with properties of GTP-binding proteins that function in transcriptional activation (Blaho and Roizman, 1991).

HSV-1 and VZV ICP4 have reportedly been associated with the tegument of purified virions (Yao and Courtney, 1989; Kinchington et al., 1992) and/or plasma membranes (Yao and Courtney, 1991), suggesting a possible adjuvant to

VP16-mediated trans-induction. However, recent evidence has challenged this view, suggesting that ICP4 is instead associated with a novel class of non-infectious enveloped tegument structures lacking capsids or DNA (L particles) common to alphaherpesviruses, including HSV-1 (McLauchlan and Rixon, 1992; Szilagyi and Cunningham, 1991).

Without exception, ICP4 genes have been found as two copies relegated to the IR_S/TR_S or R_S regions; these genes and their corresponding polypeptides, ranging in size between 170- and 200-kDa, have been identified for EHV-1 (Caughman et al., 1988; Grundy et al., 1989; Robertson et al., 1988; Telford et al., 1992), PRV (Cheung, 1989; Ihara et al., 1983), VZV (Davison and Scott, 1986; Forghani et al., 1990; Shiraki and Hyman, 1987), BHV-1 (Wirth et al., 1991), and MDV (R. Morgan, pers. comm.). Analysis of the predicted amino acid ICP4 sequences of HSV-1 and VZV have identified five highly-related sequence blocks (McGeoch et al., 1986). These domains are conserved to varying extents in PRV and VZV. EHV-1 was found to contain a similar pattern of homology, with a number of clear differences nonetheless (Grundy et al., 1989); on the other hand, regions 1, 3, and 5 were found to lack homology with PRV (Cheung, 1989).

All of the ICP4 genes are expressed as immediate-early transcripts, however, in addition to the single, spliced IE 6.0 kb transcript (Harty et al., 1989), EHV-1 contains a 3'-coterminal 4.4 kb E transcript synthesized during the E and L stages which encodes a 130-kDa polypeptide in-frame with its larger 200 kDa product (Harty and O'Callaghan, 1991). Interestingly, the BHV-1 transcript shares a promoter and a short exon with its ICP0-homologous transcript. However, its coding region is contained within the intron of the latter (Wirth et al., 1991).

Although PRV ICP4 is clearly known to *trans*-activate cellular- and heterologous viral genes, its effects on PRV gene expression are poorly understood. Transient expression assays have indicated that the ICP4s of EHV-1 and VZV can negatively autoregulate their own promoters (Disney et al., 1990; Smith et al., 1992), in addition to activating E-L promoters (Inchauspe et al., 1989; Smith et al., 1992). These are properties shared by HSV's ICP4. However, unlike HSV, VZV's ICP4 promoter appears to be negatively regulated by its own ICP0 homolog (ORF61; Nagpal and Ostrove, 1991); this lends further support to the view that mechanisms for controlling latent VZV infections differ from those of HSV (Croen et al., 1988).

2. US1 (ICP22).

In contrast to ICP4, the roles of ICP22 and ICP47 are more obscure. ICP22 homologs exhibit significant differences in length, aa conservation, and transcriptional kinetics. Compared with the ICP22 homologs of MDV (Brunovskis and Velicer, 1992a; Ross et al., 1991b), VZV (Davison and Scott, 1986), PRV (Zhang and Leader, 1990), and EHV-1 (Holden et al., 1992a; Telford et al., 1992), HSV-1 ICP22 has approximately 100 additional aa at its amino-terminal end. Length divergences are particularly pronounced in comparing the 179 aa ICP22 ORF of MDV with the 481 aa ORF of HSV-1. Apart from the others, PRV ICP22 contains a remarkable C-terminal acidic region in which nearly three-quarters of the 138 residues are either aspartic- or glutamic acid (Zhang and Leader, 1990). In contrast to their IE HSV-1 counterpart, ICP22 homologs of MDV, EHV-1, PRV, and BHV-1 exhibit strikingly different patterns in their expression kinetics. MDV US1 encodes an abundantly expressed 27-kDa late class cytoplasmic phosphoprotein, pp27 (Brunovskis and Velicer, 1992b). PRV has only one IE gene which codes for its ICP4 homolog (Cheung, 1989); failing to find immediate-early regulation of PRV US1 was therefore not a

surprise (Zhang and Leader, 1990b). The bovine- and equine ICP22 homologs are apparently regulated at *two* different levels. BHV-1 ICP22 is an abundantly expressed 55-kDa phosphoprotein, regulated at both IE and late times (M. Schwyzer, pers. comm.); EHV-1 ICP22 is expressed from both early and late promotors, resulting in independent transcripts which could code for proteins of 293- or 469 aa, respectively (Holden et al., 1992a). These recent observations appear consistent with a much earlier prediction (Honess, 1984) invoking the recombination/isomerization process to account for the possible dislocation of normal regulatory elements from genes (or their homologs) with transcripts crossing the boundaries of the repeat-unique junctions (e.g. α22, α47) thereby altering their normal gene regulation. While MDV's US1 is located entirely in its U_S region, PRV, EHV-1, BHV-1, and VZV US1 genes are found as two copies in the adjoining repeats (Figure 4). The above discrepancies in gene regulation kinetics are reminiscent of similar findings for other genes of EHV-1 (Harty and O'Callaghen, 1991), PRV (Cheung, 1991), BHV-1 (Wirth et al., 1992), and HCMV (Stenberg et al., 1989).

Although the function of US1 family genes is not yet clear, a number of interesting findings have emerged. Some of these suggest ICP22 encodes a determinant for tissue tropism. This is based on two observations. First, while ICP22 is dispensable for growth in some cell lines, ICP22 mutants grow very poorly in others. This led to the suggestion that certain cell lines (e.g. Vero) contain a host ICP22-like function which can allow for the growth of the mutant (Sears et al., 1985). These observations appear consistent with the fact that ICP22-less mutants are less virulent or capable of establishing latency in mice (Meignier et al., 1988; Roizman et al., 1982).

It should be pointed out that although HSV ICP22 is considered 'nonessential' for growth in cell culture, such a conclusion is based on the use

of a mutant which maintains the ability to express the NH_2-terminal 200 aa of HSV-1's ICP22 (Ackermann et al., 1985; Post and Roizman, 1981; McGeoch et al., 1985). This results in the expression of a 33.7-kDa phosphoprotein in HEp-2 cells and at least three distinct 33.7-39-kDa phosphoproteins in BHK cells (Ackermann et al., 1985). Like the full-length ICP22, the truncated polypeptides were similarly phosphorylated and localized to the nucleus. These truncated derivatives may be relevant to cell culture growth in view of the report that an oligo(nucleoside methylphosphonate) dodecamer derivative complementary to the splice junction of the ICP22 mRNA 4 effected a 98% decrease in HSV-1 titers with little, if any, deletarious effect on host-cell macromolecular metabolism (Kulka et al., 1989).

The functional nature of ICP22 homologs may relate to gene regulation. HSV-1 ICP22 is a predominantly nuclear protein (Fenwick et al., 1978; Fenwick et al., 1980) which is known to associate with chromatin (Hay and Hay, 1980). An HSV-1 ICP22 mutant has been characterized as defective for late gene expression (Sears et al., 1985). In another study, use of a temperature sensitive mutant in rat XC cells at the non-permissive temperature resulted in a severe reduction in L protein synthesis; this defect was correlated with a lack of ICP22 protein expression (Epstein and Jacquemont, 1983; Jacquemont et al., 1984). Although it is still too early to say whether ICP22s have any particular preference for activation or repression, BHV-1 ICP22 has been recently found to express a 55-kDa phosphoprotein inhibiting target promoters of all kinetic classes tested (M. Schwyzer, pers. comm.). The VZV ICP22 protein has been reported to repress the IE promoter of its ICP4 homolog, but stimulate the E promoter of its TK homolog (Jackers et al., 1992).

The consequences of this repression in VZV are as yet unclear, however, recent evidence suggests a potentially important role in the maintenance of a

latent growth state. By *in situ* hybridization, VZV gene 63 transcripts have been found in latently-infected neurons (Vafai et al., 1988a) or in neurons infected with VZV in vitro (Merville-Louis et al., 1992). ICP22-homologous sequences in EHV-1 defective interfering particles (DIPs) may potentially contribute to the oncogenic transformation and persistence associated with hamster embryo fibroblast infections. Interestingly, a unique recombination event has been reported to juxtapose a majority of EHV-1's ICP22 ORF in-frame with a potential zinc-finger motif derived from the C-terminal portion of its ICP27 homolog (Holden et al., 1992a; Yalamanchili et al., 1990).

3. US2

The role of this gene is completely obscure. US2-homologous genes are known to be encoded by HSV-1 (McGeoch et al., 1985), HSV-2 (McGeoch et al., 1987), VZV (Davison and Scott, 1986b), PRV (van Zijl et al., 1990), EHV-1 (Breeden et al., 1992; Colle et al., 1992; Telford et al., 1992), and MDV (Cantello et al., 1991, Ross et al., 1991, Brunovskis and Velicer, 1992a). These are the only group of S region homologs with a highly conserved N-terminus; all of which begin with the sequence M-G-V contained within a notably hydrophobic N-terminal region, possibly a signal peptide for membrane-bound translation (McGeoch et al., 1985). The highly conserved glycine residue may be a target for myristylation (Towler et al., 1987). A myristilated hydrophobic region might be expected to play a role in mediating transient membrane interactions (Schultz and Oroszlan, 1984). Specific immunological reagents have been created and used to identify the 28- and 29-kDa US2 polypeptides of PRV (van Zijl et al., 1990) and MDV (Brunovskis and Velicer, 1992c). However, further information regarding their functional or structural nature is lacking.

Specific US2 mutants, have been generated for HSV-1 (Weber et al., 1987), PRV (de Wind et al., 1990), and MDV US2 (Cantello et al., 1991); in all three cases US2 was found to be dispensable for growth in cell culture. Unlike the other U_S mutants, US2 mutants have thus far been found to maintain their virulence in animal hosts. HSV-1's US2 mutant failed to exhibit a significant loss in the ability to replicate in the central nervous system of mice (Weber et al., 1987); PRV's US2 mutant was found to maintain a comparatively normal level of virulence in its natural pig host (Kimman et al., 1992).

4. US3 (PK).

Protein kinases (PK) constitute a large and diverse family of enzymes differing in their substrate specificities, regulatory control, and the amino acids that they phosphorylate. Nevertheless, all eukaryotic PKs thus far studied show similarities in a clearly identifiable domain of about 250 aa, which contains a number of conserved subdomains important for catalytic functions (Hanks et al., 1988). McGeoch and Davison (1986) were the first to note that HSV-1 (US3, McGeoch et al., 1985) and VZV (gene 66, Davison and Scott, 1986b) shared a common gene that was related to members of the protein kinase family. Additional US3 PK-homologous genes have since been identified in HSV-2 (McGeoch et al., 1987), PRV (van Zijl et al., 1990; Zhang et al., 1990), EHV-1 (Colle et al., 1992; Telford et al., 1992), and MDV (Brunovskis and Velicer, 1992a; Ross et al, 1991). While all of these share sequences corresponding to the predicted catalytic domain, each contain an unrelated 100-200 aa NH_2-terminal region. Because these homologs are an integral and evolutionarily conserved part of their respective genetic repertoires (in contrast to oncogenic retrovirus PK genes), Leader and Purves (1988) characterized the US3 homologs as the first authentic eukaryotic viral PK genes described to date.

Further inspection has revealed that US3 PK-homologous members contain sequence motifs characteristic of serine-threonine PKs (Leader and Purves, 1988). The US3 PK family appears to define a distinct subfamily within the serine-threonine protein kinase superfamily. It is thought that related cellular counterparts exist and await future characterization (Hanks et al., 1988). Interestingly, one of three MDV US3-directed antisera was recently found to immunoprecipitate a 68-kDa cellular protein (Brunovskis and Velicer, 1992c). The interaction appeared to be specific, insofar as the fusion protein immunogen used to generate this antisera was able to block this precipitation. Further work will be necessary to determine whether this cellular protein is in fact a cellular counterpart to the US3 family of PKs.

The PRV and HSV-1 PKs were previously discovered in cytoplasmic extracts of infected hamster fibroblasts and distinguished from known cellular protein kinases (Katan et al., 1985; Purves et al., 1986a). Its enzyme was purified to near- or complete homogeneity (Purves et al, 1987a; Frame et al., 1987) and shown to have the following characteristics. (1) Constitutive activity, requiring no effector; (2) Autophosphorylation capabilities (Purves et al., 1987a; Frame et al., 1987); (3) A high KCL concentration optimum (Katan et al., 1985; Purves et al., 1986a); and (4) Transfer of phosphate from ATP to seryl or threonyl residues present in basic (but not acidic) artificial substrates (such as protamine) and in synthetic peptide substrates containing several arginyl residues on the NH_2-terminal side of serine or threonine (Katan et al., 1985; Purves et al., 1986b; Leader et al., 1991). Evidence that the HSV-1 US3 gene codes for the HSV-1 PK enzyme above was based on studies showing that; (1) an HSV-1 mutant with a US3 deletion did not exhibit the PK activity above (Purves et al., 1987b) and (2) an anti-peptide antiserum directed against a C-terminal octapeptide of the US3 ORF was found to react with highly purified PK

preparations from HSV-1-infected cells (Frame et al., 1987). Using an antiserum raised against a PRV PK fusion protein expressed in E. coli, Zhang et al. (1990) confirmed the cytoplasmic localization of PRV and HSV PK, and further demonstrated its presence in purified PRV and HSV-1 virions of which the former was shown to phosphorylate the major 112-kDa PRV virion phosphoprotein in vitro. The antisera directed against HSV-1 PK was also found to precipitate a 68/69 kDa doublet, a result analogous to the smaller PK doublet identified for MDV (Brunovskis and Velicer, 1992c).

Purves et al. (1991) have recently identified an apparently essential virion-containing phosphoprotein encoded by the UL34 gene (McGeoch et al., 1988; Purves et al., 1991) which is posttranslationally modified by HSV-1 PK. Evidence was based on the fact that site-specific mutagenesis of serine or threonine residues present in a potential US3 PK consensus target site (Leader et al., 1991; Purves et al., 1986b) led to the replacement of the 30-kDa wild-type UL34 phosphoprotein with a slower-migrating 33-kDa polypeptide of similar mobility to a novel product identified in HSV-1 PK$^-$ infected cells. Mutants altered in the PK consensus site were further characterized by greatly impaired growth properties.

Although HSV-1 and PRV PK mutants are known to grow in cell culture, altered or impaired growth properties have been cited in both cases (de Wind et al., 1990; Purves et al., 1991). PRV and HSV-1 PK mutants also show a significantly decreased virulence in pigs (Kimman et al., 1991) and mice (Meignier et al., 1988), respectively. Although the PRV mutant did not appear to be adversely affected in its tissue tropism, it was found to display an altered morphogenesis, possibly contributing to its lower replication in vitro (de Wind et al., 1990) and in vivo (Kimman et al., 1992).

8. US4 (gG).

Reflecting their close evolutionary relationship, HSV-1 and -2 U_S homologs share aa sequence identities of 70-80% (McGeoch et al., 1987). Their corresponding gG polypeptides exhibit pronounced serotype-specific differences. These differences provide the basis for serological approaches which facilitate discrimination between infections of these two serotypes (Sanchez-Martinez et al., 1991). Two groups (Marsden et al., 1984; Roizman et al., 1984) originally identified and mapped (to the U_S region) HSV serotype-2-specific glycoproteins with apparent molecular masses of 92,000 and 124,000, respectively. These polypeptides were subsequently found to represent identical glycoproteins (Balachandran and Hutt-Fletcher, 1985); the above size discrepancies were attributed to differences in the cross-linking agents used in the two gel systems. Soon thereafter, a smaller glycoprotein was mapped to a similar location in the HSV-1 genome; this suggested that the new glycoprotein was the HSV-1 equivalent of gG-2 (Ackermann et al., 1986; Richman et al., 1986). Sequence analysis of the HSV-2 US4 gene, in conjunction with the generation and use of a serotype-common anti-peptide antiserum confirmed this proposal and showed that gG-1 had approximately 400-500 aa deleted relative to gG-2 (McGeoch et al., 1987). Sequence conservation is mainly limited to the C-terminal portions of their respective proteins. Additional gG homologs have been sequenced and mapped to the U_S regions of PRV (Rea et al., 1985) and EHV-1 (Colle et al., 1992; Telford et al., 1992). In both cases, the conserved regions contain sequences homologous to the gG-2 specific portion. MDV (Brunovskis and Velicer, 1992) and VZV (Davison and Scott, 1986b) appear to lack gG-1 or gG-2 counterparts.

PRV (Rea et al., 1985) and HSV-2 (Su et al., 1987) gGs are known to undergo proteolytic processing events which result in the secretion of partial

(HSV-2)- or nearly full-length (PRV) products. However, nothing is known about these or other alphaherpesvirus gG proteins. HSV-1 (Weber et al., 1987) and PRV (de Wind et al., 1990; Mettenleiter et al., 1990b) gG mutants are not impaired in their ability to grow in cell culture. While an HSV-1 gG mutant did show a reduced ability to replicate in the central nervous system of rats (Weber et al., 1987), PRV gG mutants appeared to exhibit normal virulence in both mice (Thomsen et al., 1987) and pigs (Kimman et al., 1992). From an immunological perspective, anti-PRV gG antibodies failed to protect animals against lethal challenge (Thomsen et al., 1987), while vaccinia-gG-1 recombinants failed to induce a detectable neutralizing antibody response (Blacklaws et al., 1990).

6. US5 (gJ).

The US5 gene encodes a 92-aa ORF, apparently specific to HSV-1 and -2 (McGeoch et al., 1985; McGeoch et al., 1987). The predicted aa sequence has features characteristic of membrane-bound glycoproteins; its putative polypetide has recently been designated as gJ, although details of its characterization have not yet been published. Tn5-inserted HSV-1 US5 mutants have been reported to be unaffected for replication in cell culture or in mouse central nervous system (Weber et al., 1987).

7. US6 (gD).

Glycoprotein D (US6) is considered the only U_S region polypeptide essential for replication in cell culture. This reflects its obligate role in virus-cell penetration, initially demonstrated by work showing that; (1) anti-gD neutralizing antibodies monoclonal antibodies (MAbs) permit adsorption but inhibit penetration (Fuller and Spear, 1987; Highlander et al., 1987); and (2) an HSV mutant in which gD sequences are replaced by *B*-galactosidase sequences binds to, but is unable to penetrate into cells (Ligas and Johnson 1988). A similar role in penetration has since been demonstrated for the gD

homologs of EHV-1 (Whittaker et al., 1992), BHV-1 (Fehler et al., 1992), and PRV (Rauh and Mettenleiter, 1991; Peeters et al., 1992).

In spite of its importance to virus-cell penetration, PRV's gD is dispensable for cell-cell spread (Peeters et al., 1992; Rauh and Mettenleiter, 1991). VZV lacks such a homolog altogether. All other alphaherpesviruses thus far analyzed encode gD homologs, including HSV-1 (McGeoch et al., 1985), HSV-2 (McGeoch et al., 1987), PRV (Petrovskis et al., 1986a), EHV-1 (Audonnet et al., 1990; Flowers et al., 1991; Telford et al., 1992), BHV-1 (Tikoo et al., 1990), and MDV (Brunovskis and Velicer, 1992a, Ross et al., 1991b). Interestingly, MDV has an intact gD ORF that does not appear to be expressed in cell culture (Brunovskis and Velicer, 1992c). On the other hand, FFE cells, which support fully-productive MDV infections do appear to express this polypeptide (R. Witter, pers. comm.). These observations suggest that the cell-associated behaviour of MDV and VZV may be attributed to a lack of gD expression. Further support for this hypothesis is based on findings which show that HSV-1 gD contains a domain which restricts fusion of the envelope with cytoplasmic membranes (Campadelli-Fiume et al., 1990). Alteration of this domain promotes cytoplasmic deenvelopment of virus upon egress, resulting in the accumulation of unenveloped capsids alone or juxtaposed to cytoplasmic membranes (Campadelli-Fiume et al., 1991). Fibroblast cell cultures infected with MDV also accumulate unenveloped capsids (Nazerian et al., 1968), possibly reflecting an inability to prevent deenvelopment.

An interesting property of cell surface-expressed gD is its ability to render permissive cells resistant to further infection by homologous- or heterologous alphaherpesviruses (Chase et al., 1990; Johnson and Spear, 1989; Petrovskis et al., 1988). Such an interference phenomenon appears to represent an economical strategy for efficient viral dissemination. Johnson et al. (1990)

suggested that the interference is due to the sequestration of host receptors necessary for mediating subsequent entry. This view has been disputed by others who argue that the restriction is conferred by a specific domain within gD itself (Campadelli-Fiume et al., 1990).

Glycoprotein D is a major structural envelope component for HSV-1, -2, PRV, BHV-1, and EHV-1. This may account for the fact that it represents a primary immunogen and target for neutralizing antibodies in these different systems (Eloit et al., 1988; Para et al., 1985; Whittaker et al., 1992). In one study (Para et al., 1985), all 33 of the MAbs found to bind to purified virions of HSV were shown to immunoprecipitate one of five glycoproteins; all six MAbs exhibiting potent neutralizing activity were gD-specific. Two other anti-gD antibodies and the 26 others specific for either gB, gD, gE or gG had much less potent, if any, neutralizing activity. Anti-gD MAbs have been found to block both virus penetration (Highlander et al., 1987) and virion-induced cell fusion (Noble et al., 1983). Cattle or pigs with purified BHV-1 gD (Babiuk et al., 1987), PRV gD (Marchioli et al., 1987) or PRV gD-directed monoclonal antibodies (Marchioli et al., 1988) were found to be protected from disease. These results account for a great deal of interest in the use of gD as a potential subunit vaccine.

gDs are also important for inducing cellular immune responses. BHV-1 gD appears to be the most important glycoprotein antigen responsible for such responses (Hutchings et al., 1990). Virus vaccine vectors have increasingly gained favor in recent years because of their added ability to induce $CD4^+$- and $CD8^+$-mediated T-cell responses. Compared with other glycoprotein-expressing vectors, gD-expressing vaccinia virus recombinants were reported to induce the best neutralizing antibody titers, the best clearance of HSV from infected ears, protection from the establishment of latency in the sensory

ganglia following lethal doses of HSV (Blacklaws et al., 1990). Similar studies employing vaccinia virus-PRV recombinants showed that mice could be protected against lethal challenge following expression of PRV gD, gB or gC homologs. However, protection in their natural host required co-expression of PRV glycoproteins; PRV gD and gB were particularly effective (Riviere et al., 1992). The latter study raises concerns about overinterpreting the significance of results obtained using non-natural host models.

8. US7 (gI) and US8 (gE).

These two glycoproteins have been grouped together to reflect their close association with one another. At present, sequences have been obtained for HSV-1 (McGeoch et al., 1985), HSV-2 (McGeoch et al., 1987), VZV (Davison and Scott, 1986), PRV (Petrovskis et al., 1986b), EHV-1 (Audonnet et al., 1990; Elton et al., 1991; Telford et al., 1992), EHV-4 (Cullinane et al., 1988), and MDV (Brunovskis and Velicer, 1992a; Ross et al., 1991b). Both glycoproteins have been found to coprecipitate as a complex in HSV (Johnson and Feenstra, 1987; Johnson et al., 1988), PRV (Zuckermann et al., 1988), VZV (Vafai et al., 1988b; Vafai et al., 1989), and MDV (Chen et al., 1992) systems; in the case of VZV, these two glycoproteins are reported to share a common epitope (Vafai et al., 1988b; Vafai et al., 1989), consistent with the recent isolation of CD4-positive T-cell clones that can lyse target cells expressing either one of these glycoproteins (Huang et al., 1992).

VZV and HSV gI/gE complexes have been shown to act as Fc receptors (Johnson et al., 1988; Litwin et al., 1992). The HSV-1 Fc receptor (FcR) can exist in two forms. The gI/gE complex has a particular specificity for monomeric IgG; gE alone, for IgG complexes (Dubin et al., 1990). The HSV-1 FcR has been shown to utilize an antibody bipolar bridging mechanism (Frank and Friedman, 1989) to protect HSV-infected cells from antibody-dependent cellular cytotoxicity

(Dubin et al., 1991). MDV and PRV studies have thus far failed to associate FcR activity with gI/gE (Chen et al., 1992; Zuckermann et al., 1988).

In contrast to the FcR activities of HSV and VZV, the PRV gI/gE complex has been shown to possess a function deleterious for growth in some cell types, but not others; serial passage of PRV in CEFs invariably selects for gI and/or gE mutants which have a growth advantage not found in rabbit kidney cells (Mettenleiter, 1988b; Zuckermann et al., 1988). In conjunction with unknown cellular functions, these mutants exhibit an enhanced ability for virus release (Zsak et al., 1989).

Interestingly, nearly all of the attenuated vaccine strains used against PRV are derived from genetically engineered strains lacking gI or cell culture-attenuated strains (such as Bartha or Norden) which have undergone spontaneous deletions involving gI (Wittmann and Rziha, 1989). Extended cell culture passages of MDV and EHV-1 have also been associated with deletions and/or rearrangements involving the gI/gE region (Colle et al., 1992; unpublished observations; H.-J. Kung, pers. comm.). The latter is exemplified by the EHV-1 Kentucky A cell culture strain which contains a 3.9 kb deletion of U_S sequences coding for gI, gE, and an additional 130 aa ORF (Figure 4).

To understand the nature of attenuation as it relates to gI/gE, it is necessary to consider their function apart from a possible role in immunoevasion. PRV's gI/gE region has been implicated as a marker for neurovirulence prior to any knowledge of its genetic sequence properties (Lomniczi et al., 1984; Berns et al., 1985). These observations have been recently confirmed by work showing that a specific deletion of PRV gE significantly reduced the spread of infection in both rat (Card et al., 1992) and pig (Kimman et al., 1992) central nervous systems. It has been suggested that the neuroinvasive attributes of PRV gE result from cell-specific differences in

virus recognition mediated by distinct gE-cell receptor interactions (Card et al., 1992). Another study has correlated the presence of gE with the ability to spread in the nasal mucosa and to promote nuclear- rather than cytoplasmic envelopment (Pol et al., 1991).

Inactivation of gI or gE is known to strongly reduce virulence in pigs (Kimman et al., 1992). In contrast to pigs, PRV gI-, gE-, or gC-homolog mutants were found to essentially retain wild-type virulence levels in 1-day old chickens. on the other hand, gI/gC or gE/gC double mutants were found to have an avirulent phenotype in chickens (Mettenleiter et al., 1988a). To understand the nature of these results, it is necessary to consider recent findings which have provided important clues about the particular roles that supplementary essential genes play in the context of the host. Like gI/gE, gC is considered dispensable for growth in cell culture (Roizman and Sears, 1990). Its chief role is associated with virion-cell attachment (Herold et al., 1991; Mettenleiter et al., 1990a; Okazaki et al., 1991). Although the role of gI is not yet clear, recent evidence has implicated gE homologs in cell-cell spread (Chatterjee et al., 1989; Zsak et al., 1992). In the latter study PRV gE$^-$ mutants were found to primarily spread by the release of virus and its subsequent gC-mediated adsorption; in contrast, gC$^-$ mutants were found to spread by cell-cell transfer. Based on these observations it was argued that the avirulence associated with the gC/gI double mutants was attributed to their inability to spread by either of these two mechanisms. Further support was provided with the observation that mice passively immunized with mouse anti-PRV fared better against challenge with gE$^-$ rather than gC$^-$ mutants; this is consistent with the fact that cell-cell transfer is generally less sensitive to the effects of neutralization (Ahmed and Stevens, 1990). Since MDV is mainly disseminated by cell-cell spread, such findings

raise concerns about overinterpreting the significance of virus neutralization results which involve cell-free virus.

In animal systems, gI and gE (and other U_S genes) have been targeted as insertion sites in the creation of attenuated live vaccines (Quint et al., 1987). Pigs were found to be completely- (gE) or partially (gI) protected from lethal challenge following vaccination with these two mutants (Kimman et al., 1992). Human systems have examined gI/gE as potential subunit vaccines. Mice were recently shown to be protected from lethal challenge following vaccination with baculovirus-expressed HSV-1 gI (Ghiasi et al., 1992a) or gE (Ghiasi et al., 1992b).

gE is the most prominent antigen of VZV and is known to stimulate humoral and cell-mediated immunity and provide protection against lethal VZV challenge and establishment of latency in animals (Arvin et al., 1987). Much attention has been focused on cell-mediated immunity (CMI), partly because protection against VZV infections fail to correlate with the presence or level of specific glycoprotein-directed antibodies (Brunell et al., 1987). CMI is likely to be of particular importance for protection against cell-associated herpesviruses, such as MDV and VZV. This is emphasized by the finding that bursectomy of 1-day old chicks was not found to influence the immunity conferred by an attenuated MDV strain (Else, 1974). gI and gE may be important for CMI against MDV and VZV. Both of these glycoproteins have been reported to stimulate $CD4^+$ and $CD8^+$ T-cell clones reactive against VZV-infected cells (Arvin et al., 1991; Huang et al., 1992; Yasukawa and Zarling, 1985).

HSV and VZV gE homologs are known to undergo extensive posttranslational processing. Both contain N- and O-linked glycans and are phosphorylated, palmitylated, myristylated and sulfated (Grose, 1990; Harper and Kangro, 1990; Spear, 1984). Little is known about the functional significance

of these processing events. Recent evidence suggests that the phosphorylation of VZV gE may mediate an interaction between VZV and mannose 6-phosphate host cell receptors that is responsible for withdrawal of newly synthesized virions from the secretory pathway and their diversion to prelysosomal structures where the viral envelope is disrupted (Gabel, 1989). This was offered as a possible rationale for VZV's cell-associated nature; a similar possibility might have important implications for MDV's strict cell-associatedness.

9. US9 (10K).

HSV-1 US9 is predicted to encode a 10-kDa product (McGeoch et al., 1985). Anti-peptide antisera directed against HSV-1 US9 has been found to precipitate at least 12 related electrophoretically-distinct polypeptides ranging between 12-20-kDa in size (Frame et al., 1986). The various forms of 10K differed in abundance and phosphorylation; the lower MW forms were precipitated from virions, probably as tegument proteins associated with nucleocapsids during, or shortly following, their translocation to the nuclei of infected cells (Frame et al., 1986). The consequences of this association may be related to envelopment at the nuclear membrane. Pol et al., (1991) observed a correlation between envelopment at the nuclear membranes and the presence of PRV's US9 and gE products. Although the significance of herpesvirus envelopment at nuclear membranes has recently been questioned (Rixon et al., 1992; Whealy et al., 1991), it is interesting to note that MDV appears to lack a US9 homolog and has previously been reported to undergo cytoplasmic envelopment in the feather follicle epithelium (Johnson et al., 1975). Other homologs have been identified in PRV (Petrovskis, 1987), EHV-1 (Elton et al., 1991; Telford et al., 1992), and VZV (Davison and McGeoch, 1986; Davison and Scott, 1986)

10. US10.

HSV-1 US10 codes for a 33-kDa polypeptide identified by hybrid-selection and cell-free translation (Lee et al., 1982; Rixon and McGeoch, 1984). Its corresponding mRNA is initiated within the coding region of US11 and is translated in a leftward direction, such that it contains an unusual 110 codon out-of-frame overlap with the 3' end of US11 (Rixon and McGeoch, 1984). It has been cited as a virion protein (McGeoch et al., 1988), although no evidence of this has yet been published. A number of HSV-1 and -2 mutants with a deleted US10 (in addition to others) have been isolated and shown to promote viral growth in vitro (Brown and Harland, 1987; Longnecker and Roizman, 1986; Umene, 1986). Homologs are known to be encoded by EHV-1 (Holden et al., 1992b; Telford et al., 1992), EHV-4 (Cullinane et al., 1988) and MDV (Brunovskis and Velicer, 1992a). In examining PRV's S region gene organization (Figure 4), it appears unlikely that a US10 homolog will be found. Based on their analyis of a region highly conserved among alphaherpesviruses US10 homologs, Holden et al., (1992b) have identified a potential zinc-finger domain, possibly implicating a role in gene regulation. MDV US10 has been found to express a 25-kDa phosphoprotein that appears to be translated from an unusual bifunctional message apparently responsible for the expression of the 27-kDa MDV US1 homolog as well (Brunovskis and Velicer, 1992c). HVT appears to lack a US10 homolog (Brunovskis and Velicer, 1992c; M. Wild, pers. comm.).

12. US11.

This gene codes for an extremely basic 21-23-kDa polypeptide, originally identified by hybrid-selection (Lee et al., 1982; Rixon and McGeoch, 1984). As noted above, its C-terminal portion overlaps out-of-frame with the N-terminal region of the 33-kDa US10 polypeptide and has been further noted to contain 24 tandem repeats of the tripeptide X-P-R (Rixon and

McGeoch, 1984). Except for HSV-1 and -2, homologs have yet to be identified elsewhere. This gene has been considered a true late gene (gamma-2), whose expression is stringently dependent on viral DNA replication (Johnson et al., 1986). Although its function appears dispensable for growth in cell culture (Brown and Harland, 1987; Longnecker and Roizman, 1986; Umene, 1986), recent studies have uncovered some interesting observations regarding its functional nature.

The US11 product was originally considered a nucleolar DNA-binding protein (MacLean et al., 1987) thought to bind to 'a' sequences (Dalziel and Marsden, 1984). Recent evidence indicates that the US11 product is in fact a sequence-specific RNA-binding protein (Roller and Roizman, 1990) that negatively regulates the accumulation of a truncated transcript coding for an essential protein encoded by U_L34 (Roller and Roizman, 1991). Additional evidence has identified US11 as a virion product with a potential regulatory role that stems from a specific association with 60S ribosomal subunits (Roller and Roizman, 1992).

13. US12.

Little is known about the role of the immediate-early ICP47 product. Except for HSV-2, homologs have not been identified in other systems. Its product appears to be dispensable for growth in cell culture (Brown and Harland, 1987; Longnecker and Roizman, 1986; Mavromara-Nazos et al., 1986; Umene, 1986). The polypeptide is is not phosphorylated and is localized to the cytoplasm (Marsden et al., 1982). Transient expression assays have thus far failed to identify a role in gene regulation.

14. Non-HSV-related S region genes.

At least four non-HSV-related genes have been identified in MDV (SORF1, -2, -3, and -4; Figure 4; Brunovskis and Velicer, 1992a). Close inspection of their predicted amino acid sequences fail to have provided any obvious structural clues regarding their nature or function. SORF3 was found to exhibit significant homology to an uncharacterized fowlpox virus ORF (ORF4; Tomley et al., 1988); the other three ORFs failed to exhibit significant homologies to any protein sequences in the current databases (Brunovskis and Velicer, 1992a). Gene products for these ORFs are yet to be characterized.

Non-HSV-related genes have been identified in EHV-1 as well. These correspond to genes 67/77, 71, and 75 (Figure 4; Telford et al., 1992). Gene 71 encodes a particularly interesting ORF. Located at a position corresponding to HSV-1 gJ, the 797-aa ORF 71 contains features characteristic of glycoproteins (signal peptide, transmembrane domains, glycosylation sites etc.), yet it lacks any detectable relatedness to the smaller, 92 aa gJ sequence. Its sequence is identical to the 383-aa EUS4 ORF of the Kentucky A cell culture strain (Colle et al., 1992) at both N- and C-termini; however, compared to EUS4, the corresponding ORF of the Ab4p field isolate strain contains a unique 1242-bp in-frame insertion following position 74. It would appear that these sequences (like gI and gE) have been deleted from the Kentucky A strain following extensive serial passages. The additional 414-aa are particularly rich in serine, threonine, and alanine residues. Some of the threonine-rich sequences derive from a 225-bp region containing two different 15-bp elements repeated 6-7 times each. Mainly due to their threonine/serine/alanine-rich nature, homology searches yield several high FastA scores (over 60 scores between 100-380) that primarily reflect homologies between the additional sequences and a diverse set

of cellular proteins; such diversity complicates any obvious or meaningful functional predictions.

Gene 67/77 is present as two copies in the repeat regions and has been alternatively referred to as IR6 (Breeden et al., 1992; Holden et al., 1992b; Figure 4). Gene 76 encodes a 130-aa ORF identified by two labs (Elton et al., 1991; Telford et al., 1992) which has been deleted from the Kentucky A strain (Figure 4). ORF 76 contains residues highly conserved in EHV-4, however, the loss of one nucleotide in EHV-4 (relative to EHV-1) would lead to premature truncation (relative to EHV-1 ORF 76) resulting in a smaller 77-aa ORF. Leaving out a nucleotide in their original EHV-4 sequence (Cullinane et al., 1988) would theoretically lead to a 123-aa sequence that is 61% identical to the 130-aa ORF 76 sequence. Neither of these latter ORFs (67/77 or -76) contain any obvious structural features that would predict a possible function.

E. In conclusion.

The broad nature of this literature review provides a useful framework for thinking about MDV as an alphaherpesvirus. From this we can see that more genetic- and biological parallels appear to exist between the oncogenic MDV and other alphaherpesvirus members than perhaps previously thought. Alphaherpesvirus S region genes code for products potentially responsible for specifying the varied biological properties which distinguish alphaherpesviruses from one another and from those of other phylogenetic lineages. Moreover, they offer a particularly useful model system for studying virus-cell interactions which naturally occur in nature. As such, animal studies in a natural host offer the greatest potential for examining their functional role, significance, and association with pathogenesis. Such studies should further provide new clues about the nature of herpesvirus functions accounting for the

widespread dissemination and long evolutionary history of these ancient, well-adapted, obligate intracellular parasites.

REFERENCES

Ackermann, M., D. K. Braun, L. Pereira, and B. Roizman. 1984. Characterization of herpes simplex virus 1 α proteins 0, 4, and 27 with monoclonal antibodies. J. Virol. **52**:108-118.

Ackermann, M., M. Sarmiento, and B. Roizman. 1985. Application of antibody to synthetic peptides for characterization of the intact and truncated α22 protein specified by herpes simplex virus 1 and the R325 $\alpha 22^-$ deletion mutant. J. Virol. **56**:207-215.

Ackermann, M., R. Longnecker, B. Roizman, and L. Pereira. 1986. Identification, properties, and gene location of a novel glycoprotein specified by herpes simplex virus 1. Virology **150**:207-220.

Ahmed, R. and J. G. Stevens. 1990. Viral persistence, p. 241-246. *In* B. N. Fields et al. (ed.), Virology, 2nd ed., vol. 1. Raven Press, New York.

Allday, M. J. and D. H. Crawford. 1988. Role of epithelium in EBV persistence and pathogenesis of B-cell tumours. Lancet **i**:855-857.

Arvin, A. M., S. M. Solem, C. M. Koropchak, E. Kinney-Thomas, and S. G. Paryani. 1987. Humoral and cellular immunity to varicella-zoster virus glycoprotein gpI and to a non-glycosylated protein, p170, in the strain 2 guinea-pig. J. Gen. Virol. **68**:2449-2454.

Audonnet, J.-C., J. Winslow, G. Allen, and E. Paoletti. 1990. Equine herpesvirus type 1 unique short fragment encodes glycoproteins with homology to herpes simplex virus type 1 gD, gI and gE. J. Gen. Virol. **71**:2969-2978.

Babiuk, L. A., J. L'Italien, S. van Drunen Littel-van den Hurk, T. Zamb, M.J.P. Lawman, G. Hughes, and G. A. Gifford. 1987. Protection of cattle from bovine herpesvirus type 1 (BHV-1) infection by immunization with individual glycoproteins. Virology **159**:57-66.

Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatful, G. S. Hudson, S. C. Satchwell, C. Seguin, P. S. Tuffnell, and B. G. Barrell. 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature **310**:207-211.

Bachenheimer, S. L., E. D. Kieff, L. F. Lee, and B. Roizman. 1972. Comparative studies of DNAs of Marek's disease and herpes simplex viruses, p. 74-81. *In* P. M. Biggs, G. de-The, and L. N. Payne (eds.), Oncogenesis and Herpesviruses, IARC, Lyons, France.

Baines, J. D. and B. Roizman. 1991. The open reading frames U_L3, U_L4, U_L10, and U_L16 are dispensable for the replication of herpes simplex virus 1 in cell culture. J. Virol. **65**:938-944.

Balachandran, N. and L. M. Hutt-Fletcher. 1985. Synthesis and processing of glycoprotein gG of herpes simplex virus type 2. J. Virol. **54**:825-832.

Barahona, H., L. V. Melendez, and J. L. Melnick. 1974. A compendium of herpesviruses isolated from non-human primates. Intervirology **3**:175-192.

Barker, D. E. and B. Roizman. 1990. Identification of three genes nonessential for growth in cell culture near the right terminus of the unique sequences of long component of herpes simplex virus 1. Virology **177**:684-691.

Baumann,, R. P., D. C. Sullivan, J. Staczek, and D. J. O'Callaghan. 1986. Genetic relatedness and colinearity of genomes of equine herpesvirus types 1 and 3. J. Virol. **57**:816-825.

Beck, S. and B. G. Barrell. 1988. Human cytomegalovirus encodes a glycoprotein homologous to MHC class-I antigens. Nature **331**:269-272.

Berns, A., A. van den Ouweland, W. Quint, J. van Oirschot, and A. Gielkens. 1985. Presence of markers for virulence in the unique short region or repeat region or both of pseudorabies hybrid viruses. 1985. J. Virol. 53:89-93.

Biggs, P. M. 1985. Spread of Marek's disease, p. 329-340. *In* L. N. Payne (ed.), Marek's disease. Martinus Nijhoff, Boston.

Binns, M. M. and N.L.J. Ross. 1989. Nucleotide sequence of the Marek's disease virus (MDV) RB-1B A antigen gene and the identification of the MDV A antigen as the herpes simplex virus-1 glycoprotein C homologue. Virus Res. 12:371-382.

Bird, A. P. 1980. DNA methylation and the frequency of CpG in animal DNA. Nucl. Acids Res. 8:1499-1504.

Blacklaws, B. A., S. Krishna, A. C. Minson, and A. A. Nash. 1990. Immunogenicity of herpes simplex virus type 1 glycoproteins expressed in vaccinia virus recombinants. Virology 177:727-736.

Blaho, J. A. and B. Roizman. 1991. ICP4, the major regulatory protein of herpes simplex virus shares features common to GTP-binding proteins and is adenylated and guanylated. J. Virol. 65:3759-3769.

Bradley, G., M. Hayashi, G. Lancz, A. Tanaka, and M. Nonoyama. 1989a. Structure of the Marek's disease virus *Bam*HI-H gene family: genes of putative importance for tumor induction. J. Virol. 63:2534-2542.

Bradley, G., G. Lancz, A. Tanaka, and M. Nonoyama. 1989b. Loss of Marek's disease virus tumorigenicity is associated with truncation of RNAs transcribed within *Bam*HI-H. J. Virol. 63:4129-4135.

Braun, R. W., H. K. Teute, H. Kirchner, and K. Munk. 1984. Replication of herpes simplex virus in human T lymphocytes: characterization of the viral target cell. J. Immunol. 132:914-919.

Breeden, C. A., R. R. Yalamanchili, and D. J. O'Callaghan. 1992. DNA sequencing and transcriptional mapping of two genes encoded at the EHV-1 inverted repeat/unique short junction. Genbank accession number, M80429.

Brown, S. M. and J. Harland. 1987. Three mutants of herpes simplex virus type 2: one lacking the genes US10, US11 and US12 and two in which R_S has been extended by 6 kb to 0.91 map units with loss of U_S sequences between 0.94 and the U_S/TR_S junction. J. Gen. Virol. 68:1-18.

Browne, H., G. Smith, S. Beck, and T. Minson. 1990. A complex between the MHC class I homologue encoded by human cytomegalovirus and B_2 microblobulin. Nature 347:770-772.

Brunell, P. A., V. M. Novelli, P. M. Keller, and R. W. Ellis. 1987. Antibodies to the three major glycoproteins of varicella-zoster virus: search for the relevant host immune response. J. Infect. Dis. 156:430-435.

Brunovskis, P. and L. F. Velicer. 1992a. Structural characterization of the Marek's disease virus (MDV) unique short region: presence of alphaherpesvirus-homologous, fowlpox virus-homologous and MDV-specific genes. Submitted for publication.

Brunovskis, P. and L. F. Velicer. 1992b. Marek's disease virus expresses an unusual 27-kDa late class cytoplasmic phosphoprotein homologous to the 68-kDa herpes simplex virus 1 immediate-early nuclear phosphoprotein, ICP22. Manuscript in preparation.

Brunovskis, P. and L. F. Velicer. 1992c. Analysis of Marek's disease virus unique short region polypeptide expression with antisera to inducible, bacterially expressed fusion proteins. Manuscript in preparation.

Bryans, J. T. 1969. On immunity to disease caused by equine herpesvirus 1. J. Am. Vet. Med. Ass. 155:294-300.

Buckmaster, A. E., S. D. Scott, M. J. Sanderson, M.E.G. Boursnell, N.L.J. Ross, and M. M. Binns. 1988. Gene sequence and mapping data from Marek's disease virus and herpesvirus of turkeys: implication for herpesvirus classification. J. Gen. Virol. **69**:2033-2042.

Buscaglia, C., B. W. Calnek, and K. A. Schat. 1988. Effect of immunocompetence on the establishment and maintenance of latency with Marek's disease herpesvirus. J. Gen. Virol. **69**:1067-1077.

Buscaglia, C. and B. W. Calnek. 1988. Maintenance of Marek's disease herpesvirus latency in vitro by a factor found in conditioned medium. J. Gen. Virol. **69**:2809-2818.

Cai, W. and P. A. Schaffer. 1992. Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early, and late genes in productively infected cells. J. Virol. **66**:2904-2915.

Calnek, B. W., H. K. Adldinger, and D. E. Kahn. 1970. Feather follicle epithelium: a source of enveloped and infectious cell-free herpesvirus from Marek's disease. Avian Dis. **14**:219-233.

Calnek, B. W., K. A. Schat, L.J.N. Ross, W. R. Shek, and C.-L.H. Chen. 1982. *In vitro* infection of lymphocytes with Marek's disease virus. J. Natl. Cancer Inst. **69**:709-713.

Calnek, B. W. 1986. Marek's disease, a model for herpesvirus oncology. Crit. Rev. Microbiol. **12**:293-320.

Calnek, B. W. and R. L. Witter. 1991. Marek's disease, p. 342-385. *In* B. W. Calnek (ed.), Diseases of poultry, Iowa State University Press.

Calnek, B. W. and K. A Schat. 1991. Proliferation of chicken lymphoblastoid cells after in vitro infection with Marek's disease virus. Avian Dis. **35**:728-737.

Campadelli-Fiume, G., S. Qi, E. Avitabile, L. Foa-Tomasi, R. Brandimarti, and B. Roizman. 1990. Glycoprotein D of herpes simplex virus encodes a domain which precludes penetration of cells expressing the glycoprotein by superinfecting herpes simplex virus. J. Virol. **64**:6070-6079.

Campadelli-Fiume, G., F. Farabegoli, S. Di Gaeta, and B. Roizman. 1991. Origin of unenveloped capsids in the cytoplasm of cells infected with herpes simplex virus 1. J. Virol. **65**:1589-1595.

Cantello, J. L., A. S. Anderson, A. Francesconi, and R. W. Morgan. 1991. Isolation of a Marek's disease virus (MDV) recombinant containing the lacZ gene of Escherichia coli stably inserted within the MDV US2 gene. J. Virol. **65**:1584-1588.

Card, J. P., M. E. Whealy, A. K. Robbins, and L. W. Enquist. 1992. Pseudorabies virus envelope glycoprotein gI influences both neurotropism and virulence during infection of the rat visual system. J. Virol. **66**:3032-3041.

Carozza, J. H., T. N. Fredrickson, R. P. Prince, and R. E. Luginbuhl. 1973. Role of desquamated epithelial cells in transmission of Marek's disease. Avian Dis. **17**:767--781.

Caughman, G. B., A. T. Robertson, W. L. Gray, D. C. Sullivan, D. J. O'Callaghan. 1988. Characterization of equine herpesvirus type 1 immediate early proteins. Virology **163**:563-571.

Cebrian, J., C. Kaschka-Dierich, N. Berthelot, and P. Sheldrick. 1982. Inverted repeat nucleotide sequences in the genomes of Marek disease virus and the herpesvirus of the turkey. Proc. Natl. Acad. Sci. USA **79**:555-558.

Chase, C.C.L., K. Carter-Allen, C. Lohff, and G. J. Letchworth III. 1990. Bovine cells expressing herpesvirus 1 (BHV-1) glycoprotein IV resist infection by BHV-1, herpes simplex virus, and pseudorabies virus. J. Virol. **64**:4866-4872.

Chatterjee, S., J. Koga, and R. J. Whitley. 1989. A role for herpes simplex virus type 1 glycoprotein E in induction of cell fusion. J. Gen. Virol. **70**:2157-2162.

Chee, M. and B. Barrell. 1990. Herpesviruses: a study of parts. Trends Genet. **6**:86-91.

Chee, M. S., S. C. Satchwell, E. Preddie, K. M. Weston, and B. G. Barrell. 1990a. Human cytomegalovirus encodes three G protein-coupled receptor homologues. Nature **344**:774-777.

Chee, M. S., A. T. Bankier, S. Beck, R. Bohni, C. M. Brown, R. Cerny, T. Horsnell, C. A. Hutchison III, T. Kouzarides, J. A. Martignetti, E. Preddie, S. C. Satchwell, P. Tomlinson, K. M. Weston, and B. G. Barrell. 1990b. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. Curr. Top. Microbiol. Immunol. **154**:125-169.

Chen, X. and L. F. Velicer. 1991. Multiple bidirectional initiations and terminations of transcription in the Marek's disease virus long repeat regions. J. Virol. **65**:2445-2451.

Chen, X., P.J.A. Sondermeijer, and L. F. Velicer. 1992. Identification of a unique Marek's disease virus gene which encodes a 38-kilodalton phosphoprotein and is expressed in both lytically infected cells and latently infected lymphoblastoid tumor cells. J. Virol. **66**:85-94.

Chen, X., P. Brunovskis, and X. Chen. 1992. Identification and characterization of the Marek's disease virus homologs of the herpes simplex virus glycoproteins I and E. Submitted for publication.

Cheung, A. K. 1989. DNA nucleotide sequence analysis of the immediate-early gene of pseudorabies virus. 1989. Nucl. Acids Res. **17**:4637-4646.

Cheung, A. K. 1991. Cloning the latency gene and the early protein 0 gene of pseudorabies virus. J. Virol. **65**:5260-5271.

Cho, B. R. 1976a. In vitro biological differences between the pathogenic and apathogenic Marek's disease herpesvirus. Avian Dis. **20**:242-252.

Cho, B. R. 1976b. A possible association between plaque type and pathogenicity of Marek's disease herpesvirus. Avian Dis. **20**:324-331.

Cho, B. R. 1981. A simple in vitro differentiation between turkey herpesvirus and Marek's disease virus. Avian Dis. **25**:839-846.

Churchill, A. E., L. N. Payne, and R. C. Chubb. 1969. Immunization against Marek's disease using a live attenuated virus. Nature. **221**:744-747.

Colle III, C., C. C. Flowers, and D. O'Callaghan. 1992. Open reading frames encoding a protein kinase, homolog of glycoprotein gX of pseudorabies virus, and a novel glycoprotein map within the unique short segment of equine herpesvirus type 1. Virology **188**:545-557.

Craig, C. P. and A. J. Nahmias. 1973. Different patterns of neurologic involvement with herpes simplex virus types 1 and 2: isolation of herpes simplex virus type 2 from the buffy coat of two adults with meningitis. J. Infect. Dis. **127**:365-372.

Croen, K.D., J. M. Ostrove, L. J. Dragovic, and S. E. Straus. 1988. Patterns of gene expression are different for varicella-zoster and herpes simplex viruses. Proc. Natl. Acad. Sci. USA **85**:9773-9777.

Cui, Z., L. F. Lee, J.-L. Liu, and H.-J. Kung. 1991. Structural analysis and transcriptional mapping of the Marek's disease virus gene encoding pp38, an antigen associated with transformed cells. J. Virol. **65**:6509-6515.

Cullinane, A. A., F. J. Rixon, and A. J. Davison. 1988. Characterization of the genome of equine herpesvirus 1 subtype 2. J. Gen. Virol. **69**:1575-1590.

Dalziel, R. G. and H. S. Marsden. 1984. Identification of two herpes simplex virus type 1-induced proteins which interact specifically with the *a* sequence of herpes simplex virus DNA. J. Gen. Virol. **65**:1467-1475.

Davison, A. J. and N. M. Wilkie. 1983. Location and orientation of homologous sequences in the genomes of five herpesviruses. J. Gen. Virol. **64**:1927-1942.

Davison, A. J. and J. E. Scott. 1986. DNA sequence of the major inverted repeat in the varicella-zoster virus genome. J. Gen Virol. **66**:207-220.

Davison, A. J. and D. J. McGeoch. 1986. Evolutionary comparisons of the S segments in the genomes of herpes simplex virus type 1 and varicella-zoster virus. J. Gen. Virol. **67**:597-611.

Davison, A. J. and J. E. Scott. 1986. The complete DNA sequence of varicella-zoster virus. J. Gen. Virol. **67**:1759-1816.

Davison, A. J. and P. Taylor. 1987. Genetic relations between varicella-zoster virus and Epstein-Barr virus. J. Gen. Virol. **68**:1067-1079.

Davison, A. J. 1992. Channel catfish virus: a new type of herpesvirus. Virology. **186**:9-14.

DeLuca, N. A. and P. A. Schaffer. 1988. Physical and functional domains of the herpes simplex virus transcriptional regulatory protein ICP4. J. Virol. **62**:732-743.

de Wind, N., A. Zijderveld, K. Glazenburg, A. Gielkens, and A. Berns. 1990. Linker insertion mutagenesis of herpesviruses: inactivation of single genes within the U_S region of pseudorabies virus. J. Virol. **64**:4691-4696.

Disney, G. H., T. A. McKee, C. M. Preston, and R. D. Everett. 1990. The product of varicella-zoster virus gene 62 autoregulates its own promoter. J. Gen. Virol. **71**:2999-3003.

Dubin, G., I. Frank, and H. M. Friedman. 1990. Herpes simplex virus type 1 encodes two Fc receptors which have different binding characteristics for monomeric immunoglobulin G (IgG) and IgG complex. J. Virol. **64**:2725-2731.

Dubin, G., E. Socolof, I. Frank, and H. M. Friedman. 1991. Herpes simplex virus type 1 Fc receptor protects infected cells from antibody-dependent cellular cytotoxicity. J. Virol. **65**:7046-7050.

Dutch, R. E., R. C. Bruckner, E. S. Mocarski, and I. R. Lehman. 1992. Herpes simplex virus type 1 recombination: role of DNA replication and viral *a* sequences. J. Virol. **66**:277-285.

Efstathiou, S., Y. M. Ho, S. Hall, C. J. Styles, S. D. Scott, and U. A. Gompels. 1990. Murine herpesvirus 68 is genetically related to the gammaherpesviruses Epstein-Barr virus and herpesvirus saimiri. J. Gen. Virol. **71**:1365-1372.

Eloit, M., D. Fargeaud, P. Vannier, B. Toma. 1988. Identification of the pseudorabies virus gp50 as a major target of neutralizing antibodies. Arch. Virol. **99**:45-56.

Else, R. W. 1974. Vaccinal immunity to Marek's disease in bursectomised chickens. Vet. Rec. **95**:182-187.

Elton, D. M., I. W. Halliburton, R. A. Killington, D. M. Meredith, and W. A. Bonass. 1991. Sequence analysis of the 4.7-kb BamHI-EcoRI fragment of the equine herpesvirus type-1 short unique region. Gene **101**:203-208.

Ensoli, B., P. Lusso, F. Schachter, S. F. Josephs, J. Rappaport, F. Negro, R. C. Gallo, and F. Wong-Staal. 1989. Human herpesvirus-6 increase HIV-1 expression in co-infected T cells via nuclear factors binding to the HIV-1 enhancer. EMBO J. **8**:3019-3027.

Epstein, A. L. and B. Jacquemont. 1983. Virus polypeptide synthesis induced by herpes simplex virus in non-permissive rat XC cells. J. Gen. Virol. **64**:1499-1508.

Epstein, M. A., B. G. Achong, and Y. M. Barr. 1964. Virus particles in cultured lymphoblasts from Burkitt's lymphoma. Lancet **1**:702-703.

Everett, R. D. 1987. The regulation of transcription of viral and cellular genes by herpesvirus immediate-early gene products. Anticancer Research **7**:589-604.

Everett, R. D., M. Elliott, G. Hope, and A. Orr. 1991. Purification of the DNA binding domain of herpes simplex virus type 1 immendiate-early protein Vmw175 as a homodimer and extensive mutagenesis of its DNA recognition site. Nucl. Acids Res. **19**:4901-4908.

Fabricant, C. G. 1985. Atherosclerosis: the consequence of infection with a herpesvirus. Adv. Vet. Sci. Comp. Med. **30**:39-66.

Fehler, F., J. M. Hermann, A. Saalmuller, T. C. Mettenleiter, and G. M. Keil. 1992. Glycoprotein IV of bovine herpesvirus 1-expressing cell line complements and rescues a conditionally lethal viral mutant. J. Virol. **66**:831-839.

Fenwick, M., M. Walker, and J. M. Petkevich. 1978. On the association of viral proteins with the nuclei of cells infected with herpes simplex virus. J. Gen. Virol. **39**:519-529.

Fenwick, M., M. Walker, and L. Marshall. 1980. Some characteristics of an early protein (ICP22) synthesized in cells infected with herpes simplex virus. J. Gen. Virol. **47**:333-341.

Ficken, M. D., M. P. Nasisse, G. D. Boggan, J. S. Guy, D. P. Wages, R. L. Witter, J. K. Rosenberger, and R. M. Nordgren. 1991. Marek's disease virus isolates with unusual tropism and virulence for ocular tissues: clinical findings, challenge studies and pathological features. Avian Pathol. **20**:461-474.

Flowers, C. C., E. M. Eastman, and D. J. O'Callaghan. 1991. Sequence analysis of a glycoprotein D gene homolog within the unique short segment of the EHV-1 genome. Virology. **180**:175-184.

Forghani, B., R. Mallingham, A. Vafai, J. W. Hurst, and K. W. Dupuis. 1990. Monoclonal antibody to the immediate-early protein encoded by varicella zoster virus gene 62. Virus Res. **16**:195-210.

Frame, M. C., D. J. McGeoch, F. J. Rixon, A. C. Orr, and H. S. Marsden. 1986. The 10K virion phosphoprotein encoded by gene US9 from herpes simplex virus type 1. Virology. **150**:321-332.

Frame, M. C., F. C. Purves, D. J. McGeoch, H. S. Marsden, and D. P. Leader. 1987. Identification of the herpes simplex virus protein kinase as the product of viral gene US3. J. Gen. Virol. **68**:2699-2704.

Frank, I., and H. M. Friedman. 1989. A novel function of the herpes simplex virus type 1 Fc receptor: participation in bipolar bridging of antiviral immunoglobulin G. J. Virol. **63**:4479-4488.

Frenkel, N., E. C. Schirmer, L. S. Wyatt, G. Katsafanas, E. Roffman, R. M. Danovich, and C. H. June. 1990. Isolation of a new herpesvirus from human $CD4^+$ T cells. Proc. Natl. Acad. Sci. USA **87**:748-752.

Fukuchi, K., M. Sudo, Y.-S. Lee, A. Tanaka, and M. Nonoyama. 1985. Structure of Marek's disease virus DNA: detailed restriction enzyme map. J. Virol. **51**:102-109.

Fuller, A. O. and P. G. Spear. 1987. Anti-glycoprotein D antibodies that permit adsorption but block infection by herpes simplex virus 1 prevent virion-cell fusion at the cell surface. Proc. Natl. Acad. Sci. USA **84**:5454-5458.

Gabel, C. A., L. Dubey, S. P. Steinberg, D. Sherman, M. D. Gershon, and A. A. Gershon. 1989. Varicella-zoster virus glycoprotein oligosaccharides are phosphorylated during posttranslational maturation. J. Virol. **63**:4264-4276.

Gallatin, W. M. and B. M. Longnecker. 1979. Expression of genetic resistance to an oncogenic herpesvirus at the target cell level. Nature. **280**:587-589.

Garcia-Blanco, M. A. and B. R. Cullen. 1991. Molecular basis of latency in pathogenic human viruses. Science **254**:815-820.

Gentry, G. A., M. Lowe, G. Alford, and R. Nevins. 1988. Sequence analyses of herpesviral enzymes suggest an ancient origin for human sexual behavior. Proc. Natl. Acad. Sci. USA **85**:2658-2661.

Ghiasi, H., R. Kaiwar, A. B. Nesburn, and S. L. Wechsler. 1992a. Expression of hereps simplex virus type 1 glycoprotein I in baculovirus: preliminary biochemical characterization and protection studies. J. Virol. **66**:2505-2509.

Ghiasi, H., R. Kaiwar, A. B. Nesburn, S. Slanina, and S. L. Wechsler. 1992b. Baculovirus-expressed glycoprotein E (gE) of herpes simplex virus type-1 (HSV-1) protects mice against lethal intraperitoneal and lethal ocular HSV-1 challenge. Virology **188**:469-476.

Gompels, U. A., M. A. Craxton, and R. W. Honess. 1988. Conservation of gene organization in the lymphotropic herpesviruses herpesvirus saimiri and Epstein-Barr virus. J. of Virol. **62**:757-767.

Gratama, J. W., M.A.P. Oosterveer, F. E. Zwaan, J. Lepoutre, G. Klein, and J. Ernberg. 1988. Eradication of Epstein-Barr virus by allogeneic bone marrow transplantatation: implications for the site of viral latency. Proc. Natl. Acad. Sci. USA **85**:8693-8699.

Grose, C. 1982. Varicella-zoster infections: chickenpox (varicella) and shingles (zoster), p. 85-150. *In* Glaser, R. and T. Gotlieb-Stematsky (eds.), Human herpesvirus infections. Marcel Dekker Inc., New York.

Grose C. 1990. Glycoproteins encoded by varicella-zoster virus: biosynthesis, phosphorylation, and intracellular trafficking. Annu. Rev. Microbiol. **44**:59-80.

Grundy, F. J., R. P. Baumann, and D. J. O'Callaghan. 1989. DNA sequence and comparative analyses of the equine herpesvirus type 1 immediate early gene. Virology **172**:223-236.

Hajjar, D. P. 1991. Viral pathogenesis of atherosclerosis: impact of molecular mimicry and viral genes. Am J. Pathol. **139**:1195-1211.

Hall, J. D., P. A. Furman, M. H. St. Clair, and C. W. Knopf. 1985. Reduced *in vivo* mutagenesis by mutant herpes simplex DNA polymerase involves improved nucleotide selection. Proc. Natl. Acad. Sci. USA **82**:3889-3893.

Hanks, S. K., A. M. Quinn, and T. Hunter. 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science **241**:42-52.

Harper, D. R. and H. O. Kangro. 1990. Lipoproteins of varicella-zoster virus. J. Gen. Virol. **71**:459-463.

Harris, R. A., R. D. Everett, X. Zhu, S. Silverstein, and C. M. Preston. 1989. Herpes simplex type 1 immediate-early protein Vmw110 reactivates latent herpes simplex virus type 2 in an in vitro latency system. J. Virol. **63**:3513-3515.

Harty, R. N., C. F. Colle, F. J. Grundy, and D. J. O'Callaghan. 1989. Mapping the termini and intron of the spliced immediate-early transcript of equine herpesvirus 1. J. Virol. **63**:5101-5110.

Harty, R. N. and D. J. O'Callaghan. 1991. An early gene maps within and is 3' coterminal with the immediat'e-early gene of equine herpesvirus 1. J. Virol. **65**:3829-3838.

Hay, R. T. and J. Hay. 1980. Properties of herpesvirus-induced "immediate-early" polypeptides. Virology **104**:230-234.

Hayes, M. K. and D. L. Rock. 1990. Identification of a novel bovine herpesvirus type 1 immediate-early infected cell protein. Arch. Virol. **112**:291-300.

Herold, B. C., D. WuDunn, N. Soltys, and P. G. Spear. 1991. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. J. Virol. **65**:1090-1098.

Highlander, S. L., S. L. Sutherland, P. J. Gage, D. C. Johnson, M. Levine, and J. C. Glorioso. 1987. Neutralizing monoclonal antibodies specific for herpes simplex glycoprotein D inhibit virus penetration. J. Virol. **61**:3356-3364.

Holden, V. R., R. R. Yalamanchili, R. N. Harty, and D. J. O'Callaghan. 1992a. ICP22 homolog of equine herpesvirus 1: expression from early and late promoters. J. Virol. **66**:664-673.

Holden, V. R., R. R. Yalamanchili, R. N. Harty, and D. J. O'Callaghan. 1992b. Identification and characterization of an equine herpesvirus 1 late gene encoding a potential zinc finger. Virology **188**:704-713.

Honess, R. W. and B. Roizman. 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. **14**:8-19.

Honess, R. W. and D. H. Watson. 1977. Unity and diversity in the herpesviruses. J. Gen. Virol. **37**:15-37.

Honess, R. W. 1984. Herpes simplex and 'the herpes complex': diverse observations and a unifying hypothesis. J. Gen. Virol. **65**:2077-2107.

Honess, R. W., U. A. Gompels, B. G. Barrell, M. Craxton, K. R. Cameron, R. Staden, Y.-N. Chang, and G. S. Hayward. 1989. Deviations from expected frequencies of CpG dinucleotides in herpesvirus DNAs may be diagnostic of differences in the states of their latent genomes. J. Gen. Virol. **70**:837-855.

Huang, Z., A. Vafai, J. Lee, R. Mahalingam, and A. R. Hayward. 1992. Specific lysis of targets expressing varicella-zoster virus gpI or gpIV by $CD4^+$ human T-cell clones. J. Virol. 66:2664-2669.

Hughes, R.A.C. 1990. Animal models of Guillain-Barre syndrome, p. 247-259. *In* R.A.C. Hughes (ed.), Guillain-Barre syndrome. Springer-Verlag, New York-London.

Hummeler, K., G. Henle, and W. Henle. 1966. Fine structure of a virus in cultured lymphoblasts from Burkitt's lymphoma. J. Bact. **91**:1366.

Hutchings, D. L., S. van Drunen Littel-van den Hurk, and L. A. Babiuk. 1990. Lymphocyte proliferative responses to separated bovine herpesvirus 1 proteins in immune cattle. J. Virol. **64**:5114-5122.

Igarashi, T., M. Takahashi, J. Donovan, J. Jessip, M. Smith, K. Hirai, A. Tanaka, and M. Nonoyama. 1987. Restriction enzyme map of herpesvirus of turkey DNA and its collinear relationship with Marek's disease virus DNA. Virology. **157**:351-358.

Ihara, S., L. Feldman, S. Watanabe, and T. Ben-Porat. 1983. Characterization of the immediate-early functions of pseudorabies virus. Virology **131**:437-454.

Ikuta, K., K. Nakajima, M. Naito, S. H. Ann, S. Ueda, S. Kato, and K. Hirai. 1985. Identification of Marek's disease virus-specific antigens in Marek's disease lymphoblastoid cell lines using monoclonal antibody against virus-specific phosphorylated polypeptides. Int. J. Cancer **35**:257-264.

Ikuta, K., K. Nakajima, A. Kanamori, K. Maotani, J. S. Mah, S. Ueda, S. Kato, M. Yoshida, S. Nii, M. Naito, C. Nishida-Umehara, M. Saski, and K. Hirai. 1987. Establishment and characterization of a T-lymphoblastoid cell line MDCC-MTB1 derived from chick lymphocytes infected in vitro with Marek's disease serotype 1. Int. J. Cancer. **39**:514-520.

Imai, K., N. Yuasa, S. Kobayashi, K. Nakamura, K. Tsukamoto, and H. Hihara. 1990. Isolation of Marek's disease virus from Japanese quail with lymphoproliferative disease. Avian Pathol. **19**:119-129.

Inchauspe, G., S. Nagpal, and J. M. Ostrove. 1989. Mapping of two varicella-zoster virus-encoded genes that activate the expression of viral early and late genes. Virology **173**:700-709.

Iwata, A., S. Ueda, A. Ishihama, and K. Hirai. 1992. Sequence determination of cDNA clones of transcripts from the tumor-associated region of the Marek's disease virus genome. Virology **187**:805-808.

Jackers, P., P. Defechereux, L. Baudoux, C. Lambert, M. Massaer, M.-P. Merville-Louis, B. Rentier, and J. Piette. 1992. Characterization of regulatory functions of the varicella-zoster virus gene 63-encoded protein. J. Virol. **66**:3899-3903.

Jacquemont, B., B. Verrier, A. L. Epstein, and L. Machuca. 1984. Expression of immediate-early genes in herpes simplex virus type 1-infected XC cells: lack of ICP22 (68K) polypeptide. J. Gen. Virol. **65**:1331-1340.

Johnson, E. A., C. N. Burke, T. N. Fredrickson, and R. A. DiCapua. 1976. Morphogenesis of Marek's disease virus in feather follicle epithelium. J. Natl. Cancer Inst. **56**:89-99.

Johnson, P. A., C. MacLean, H. S. Marsden, R. G. Marsden, R. G. Dalziel, and R. D. Everett. 1986. The product of gene US11 of herpes simplex virus type 1 is expressed as a true late gene. J. Gen. Virol. **67**:871-883.

Johnson, D. C., and V. Feenstra. 1987. Identification of a novel herpes simplex virus type 1-induced glycoprotein which complexes with gE and binds immunoglobulin. J. Virol. **61**:2208-2216.

Johnson, D. C., M. C. Frame, M. W. Ligas, A. M. Cross, and N. D. Stow. 1988. Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral glycoproteins, gE and gI. J. Virol. **62**:1347-1354.

Johnson, R. M. and P. G. Spear. 1989. Herpes simplex virus glycoprotein D mediates interference with herpes simplex virus infection. J. Virol. **63**:819-827.

Johnson, D. C., R. L. Burke, and T. Gregory. 1990. Soluble forms of herpes simplex virus glycoprotein D bind to a limited number of cell surface receptors and inhibit virus entry into cells. J. Virol. **64**:2569-2576.

Jones, D., L. Lee, J.-L. Liu, H.-J. Kung, and J. K. Tillotson. 1992. Marek's disease virus encodes a basic-leucine zipper gene resembling the *fos/jun* oncogenes that is highly expressed in lymphoblastoid tumors. Proc. Natl. Acad. Sci. USA **89**:4042-4046.

Jones, T. R., V. P. Muzithras, and Y. Gluzman. 1991. Replacement mutagenesis of the human cytomegalovirus genome: US10 and US11 gene products are nonessential. J. Virol. **65**:5860-5872.

Jones, T. R. and V. P. Muzithras. 1992. A cluster of dispensable genes within the human cytomegalovirus genome short component: IRS1, US1 through US6, and the US6 family. J. Virol. **66**:2541-2546.

Kanamori, A., K. Ikuta, S. Ueda, S. Kato, and K. Hirai. 1987. Methylation of Marek's disease virus DNA in chicken T-lymphoblastoid cell lines. J. Gen. Virol. **68**:1485-1490.

Karlin, S., B. E. Blaisdell, and G. Schachtel. 1990. Contrasts in codon usage of latent versus productive genes of Epstein-Barr virus: data and hypotheses. J. Virol. **64**:4264-4273.

Katan, M., W. Stevely, and D. P. Leader. 1985. Partial purification and characterization of a new phosphoprotein kinase from cells infected with pseudorabies virus. Eur. J. Biochem. **152**:57-65.

Kato, S., K. Ono, M. Naito, and S. Tanabe. 1972. Immunological studies on Marek's disease virus and Epstein-Barr virus, p.485-488. *In* P. M. Biggs, G. de-The, and L. N. Payne (eds.), Oncogenesis and herpesviruses. IARC Scientific Publications No. 2, Lyon, France.

Kawamura, M., M. Hayashi, T. Furuichi, M. Nonoyama, E. Isogai, and S. Namioka. 1991. The inhibitory effects of oligonucleotides, complementary to Marek's disease virus mRNA transcribed from the BamHI-H region, on the proliferation of transformed lymphoblastoid cells, MDCC-MSB-1. J. Gen. Virol. **72**:1105-1111.

Kenzy, S. G., B. R. Cho, and Y. Kim. 1973. Oncogenic Marek's disease herpesvirus in avian encephalitis (temporary paralysis). J. Natl. Cancer Inst. **51**:977-982.

Kieff, E. and D. Liebowitz. 1990. Epstein-Barr virus and its replication, p. 1889-1920. *In* B. N. Fields, et al. (ed.), Virology, 2nd ed., vol. 2. Raven Press, New York.

Killeen, A. M., L. Harrington, L.V.M. Wall, and D. C. Kelly. 1992. Nucleotide sequence analysis of a homologue of herpes simplex virus type 1 gene US9 found in the genome of simian herpes B virus. J. Gen. Virol. **73**:195-199.

Kimman, T. G., N. de Wind, N. Oei-Lie, J.M.A. Pol, A. J. Berns, and A.L.J. Gielkens. 1992. Contribution of single genes within the unique short region of Aujeszky's disease virus (suid herpesvirus type 1) to virulence, pathogenesis and immunogenicity. J. Gen Virol. **73**:243-251.

Kinchington, P. R., L. K. Hougland, A. M. Arvin, W. T. Ruyechan, and J. Hay. 1992. The varicella-zoster virus immediate-early protein IE62 is a major component of virus particles. J. Virol. **66**:359-366.

Kishi, M., H. Harada, M. Takahashi, A. Tanaka, M. Hayashi, M. Nonoyama, S. F. Josephs, A. Buchbinder, F. Schachter, D. V. Ablashi, F. Wong-Staal, S. Z. Salahuddin, and R. C. Gallo. 1988. A repeat sequence, GGGTTA, is shared by DNA of human herpesvirus 6 and Marek's disease virus. J. Virol. **62**:4824-4827. \

Klein, G. 1972. A summing up, p. 501-515. *In* P. M. Biggs, G. de-The, and L. N. Payne (ed.), Oncogenesis and herpesviruses. IARC Scientific Publ. No. 2, Lyon, France.

Klein, G. 1989. Viral latency and transformation: the strategy of Epstein-Barr virus. Cell **58**:5-8.

Kolbert-Jons, A., E. Bogner, and K. Radsak. 1991. A 15-kilobase-pair region of the human cytomegalovirus genome which includes US1 through US13 is dispensable for growth in cell culture. J. Virol. **65**:5184-5189.

Kouzarides, T., A. T. Bankier, S. C. Satchwell, K. Weston, P. Tomlinson, and B. G. Barrell. 1987. Large-scale rearrangement of homologous regions in the genomes of HCMV and EBV. Virology **157**:397-413.

Kulka, M., C. C. Smith, L. Aurelian, R. Fishelevich, K. Meade, P. Miller, and P.O.P. Ts'O. 1989. Site specificity of the inhibitory effects of oligo(nucleoside methylphosphonate)s complementary to the acceptor splice junction of herpes simplex virus type 1 immediate early mRNA 4. Proc. Natl. Acad. Sci. USA. **86**:6868-6872.

Kupershmidt, S., G. F. Rall, Z. Lu, and T. Ben-Porat. 1992. Cleavage of concatameric DNA at the internal junction of "translocation" mutants of pseudorabies virus and inversion of their L component appear to be linked. Virology **187**:223-232.

Lawrence, G. L., M. Chee, M. A. Craxton, U. A. Gompels, R. W. Honess, and B. G. Barrell. 1990. Human herpesvirus 6 is closely related to human cytomegalovirus. J. Virol. **64**:287-299.

Leader, D. P. and F. C. Purves. 1988. The herpesvirus protein kinase: a new departure in protein phosphorylation? Trends Biochem. Sci. **13**:244-246.

Leader, D. P., A. D. Deana, F. Marchiori, F. C. Purves, and L. A. Pinna. 1991. Further definition of the substrate specificity of the alph-herpesvirus protein kinase and comparison with protein kinases A and C. Biochim. Biophys. Acta **1091**:426-431.

Lee, G. T.-Y., M. F. Para, and P. G. Spear. 1982. Location of the structural genes for gD and gE and for other polypeptides in the S component of herpes simplex virus type 1 DNA. J. Virol. **43**:41-49.

Lee, L. F., B. Roizman, P. G. Spear, E. D. Kieff, B. R. Burmester, and K. Nazerian. 1969. Marek's disease herpesvirus: a cytomegalovirus? Proc. Natl. Acad. Sci. USA **64**:951-956.

Lee, L. F., X. Liu, and R. L. Witter. 1983. Monoclonal antibodies with specificity for three different serotypes of Marek's disease viruses in chickens. J. Immunol. **130**:1003-1006.

Ligas, M. W. and D. C. Johnson. 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by -Galactosidase sequences binds to but is unable to penetrate into cells. J. Virol. **62**:1486-1494.

Lillycrop, K. A., C. L. Dent, S. C. Wheatley, M. N. Beech, N. N. Ninkina, J. N. Wood, and D. S. Latchman. 1991. The octamer-binding protein Oct-2 represses HSV immediate-early genes in cell lines derived from latently infectable sensory neurons. Neuron **7**:381-390.

Lindquester, G. J. and P. E. Pellett. 1991. Properties of the human herpesvirus 6 strain Z29 genome: G+C content, length, and presence of variable-length directly repeated terminal sequence elements. Virology **182**:102-110.

Litwin, V., W. Jackson, and C. Grose. 1992. Receptor properties of two varicella-zoster virus glycoproteins gpI and gpIV, homologous to herpes simplex virus gE and gI. J. Virol. **66**:3643-3651.

Lomniczi, B., S. Watanabe, T. Ben-Porat, and A. S. Kaplan. 1984. Genetic basis of the neurovirulence of pseudorabies virus. J. Virol. **52**:198-205.

Lomniczi, B., A. Gielkens, I. Csobai, and T. Ben-Porat. 1987. Evolution of pseudorabies virions containing genomes with an invertible long component after repeated passage in chicken embryo fibroblasts. J. Virol. **61**:1772-1780.

Longnecker, R. and B. Roizman. 1986. Generation of an inverting herpes simplex virus 1 mutant lacking the L-S junction *a* sequences, an origin of DNA synthesis, and several genes including those specifying glycoprotein E and the α47 gene. J. Virol. **58**:583-591.

Longnecker, R. S. and B. Roizman. 1987. Clustering of genes dispensable for growth in culture in the S component of the HSV-1 genome. Science **236**:573-576.

Longnecker, R., S. Chatterjee, R. J. Whitley, and B. Roizman. 1987. Identification of a herpes simplex virus 1 glycoprotein gene within a gene cluster dispensable for growth in cell culture. Proc. Natl. Acad. Sci. USA **84**:4303-4307.

Lopez, C., P. Pellett, J. Stewart, C. Goldsmith, K. Sanderlin, J. Black, D. Warfield, and P. Feorino. 1988. Characteristics of human herpesvirus-6. J. Infect. Dis. 157:1271-1273.

Lu, Z., M. DeMarchi, L. Harper, G. F. Rall, and T. Ben-Porat. 1989. Nucleotide sequences at the recombinational junctions present in pseudorabies virus variants with an invertible L component. J. Virol. 63:2690-2698.

MacLean, C. A., F. J. Rixon, and H. S. Marsden. 1987. The products of gene U_S11 of herpes simplex virus type 1 are DNA-binding and localize to the nucleoli of infected cells. J. Gen. Virol. 68:1921-1937.

Maotani, K., A. Kanamori, K. Ikuta, S. Ueda, S. Kato, and K. Hirai. 1986. Amplification of a tandem repeat within inverted repeats of Marek's disease virus DNA during serial in vitro passage. J. Virol. 58:657-660.

Marchioli, C. C., R. Y. Yancey, Jr., E. A. Petrovskis, J. G. Timmins, and L. E. Post. 1987. Evaluation of pseudorabies virus glycoprotein gp50 as vaccine for Aujeszky's disease in mice and swine: expression by vaccinia virus and chinese hamster ovary cells. J. Virol. 61:3977-3982.

Marchioli, C. C., R. Y. Yancey, Jr., J. G. Timmins, L. E. Post, B. R. Young, and D. A. Povendo. 1988. Protection of mice and swine from pseudorabies virus-induced mortality by administration of pseudorabies virus-specific mouse monoclonal antibodies. Am. J. Vet. Res. 49:860-864.

Marek, J. 1907. Multiple Nerventzuendung (Polyneuritis) bei Huehnern. Dtsch. Tieraerztl. Wochenschr. 15:417-421.

Marsden, H. S., J. Lang, A. J. Davison, R. G. Hope, and D. M. MacDonald. 1982. Genomic location and lack of phosphorylation of the HSV immediate-early polypeptide IE12. J. Gen. Virol. 62:17-27.

Marsden, H. S., N. D. Stow, V. G. Preston, M. C. Timbury, and N. M. Wilkie. 1984. Characterization of the 92,000-dalton glycoprotein induced by herpes simplex virus type 2. J. Virol. 50:547-554.

Martin, M.E.D., B. J. Thompson, R. W. Honess, M. A. Craxton, U. A. Gompels, M.-Y. Liu, E. Littler, J. R. Arrand, L. Teo, and M. D. Jones. 1991. The genomes of human herpesvirus 6: maps of unit-length and concatameric genomes for nine restriction endonucleases. J. Gen. Virol. 72:157-168.

Mavromara-Nazos, P., M. Ackermann, and B. Roizman. 1986. Construction and properties of a viable herpes simplex virus 1 recombinant lacking coding sequences of the α47 gene. J. Virol. 60:807-812.

McGeoch, D. J., A. Dolan, S. Donald, and F. J. Rixon. 1985. Sequence determination and genetic content of the short unique region in the genome of herpes simplex virus type 1. J. Mol. Biol. 181:1-13.

McGeoch, D. J., A. Dolan, S. Donald, and D. H. K. Brauer. 1986. Complete DNA sequence of the short repeat region in the genome of herpes simplex virus type 1. Nucl. Acids Res. 14:1727-1745.

McGeoch, D. J. and A. Davison. 1986. Alphaherpesviruses possess a gene homologous to the protein kinase gene family of eukaryotes and retroviruses. Nucl. Acids Res. 14:1765-1777.

McGeoch, D. J. 1987. The genome of herpes simplex virus: structure, replication and evolution. J. Cell Sci. (Suppl.) 7:67-94.

McGeoch, D. J., H.W.M. Moss, D. McNab, and M. C. Frame. 1987. DNA sequence and genetic content of the HindIII L region in the short unique component of the herpes simplex virus type 2 genome: identification of the gene encoding glycoprotein G, and evolutionary comparisons. J. Gen. Virol. 68:19-38.

McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor. 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69**:1531-1574.

McGeoch, D. J. 1990. Evolutionary relationships of virion glycoprotein genes in the S region of alphaherpesvirus genomes. J. Gen. Virol. **71**:2361-2367.

McLauchlan, J. and F. J. Rixon. 1992. Characterization of ewnveloped tegument structures (L particles) produced by alphaherpesviruses: integrity of the tegument does not depend on the presence of capsid or envelope. J. Gen. Virol. **73**:269-276.

Meignier, B., R. Longnecker, P. Mavromara-Nazos, A. E. Sears, and B. Roizman. 1988. Virulence of and establishment of latency by genetically engineered deletion mutants of herpes simplex virus 1. Virology **162**:251-254.

Melnick, J. L., M. Midulla, I. Wimberly, J. G. Barrera-Oro, and B. M. Levy. 1964. A new member of the herpesvirus group isolated from South American marmosets. J. Immunol. **92**:596-601.

Merville-Louis, M.-P., C. Sadzot-Delvaux, P. Delree, J. Piette, G. Moonen, and B. Rentier. Varicella-zoster infection of adult rat sensory neurons in vitro. J. Virol. **63**:3155-3160.

Mettenleiter, T. C., C. Schreurs, F. Zuckerman, T. Ben-Porat, and A. S. Kaplan. 1988a. Role of glycoprotein gIII of pseudorabies virus in virulence. J. Virol. **62**:2712-2717.

Mettenleiter, T. C., B. Lomniczi, N. Sugg, C. Schreurs, and T. Ben-Porat. 1988b. Host cell-specific advantage of pseudorabies virus with a deletion in the genome sequences encoding a structural glycoprotein. J. Virol. **62**:12-19.

Mettenleiter, T. C., Zsak, L., F. Zuckermann, N. Sugg, H. Kern, and T. Ben-Porat. 1990a. Interaction of glycoprotein gIII with a cellular heparinlike substance mediates adsorption of pseudorabies virus. J. Virol. **64**:278-286.

Mettenleiter, T. C., H. Kern, and I. Rauh. 1990b. Isolation of a viable herpesvirus (pseudorabies virus) mutant specifically lacking all four known nonessential glycoproteins. Virology **179**:498-503.

Metzler, D. W. and K. W. Wilcox. 1985. Isolation of herpes simplex virus regulatory protein ICP4 as a homodimeric complex. J. Virol. **55**:329-337.

Miller, G. 1990. The switch between latency and replication of Epstein-Barr virus. J. Infect. Dis. **161**:833-844.

Mocarski, E. S. and B. Roizman. 1982. Structure and role of the herpes simplex virus DNA termini in inversion, circularization and generation of virion DNA. Cell **31**:89-97.

Nagpal, S. and J. M. Ostrove. 1991. Characterization of a potent varicella-zoster virus-encoded trans-repressor. J. Virol. **65**:5289-5296.

Nahmias, A. J. 1972. Herpesviruses from fish to man- a search for pathobiologic unity. Pathobiol. Annu. **2**:153-182.

Nahmias, A. J. and B. Roizman. 1973. Infection with herpes-simplex virus 1 and 2. N. Engl. J. Med. **289**:781-789.

Nakajima, K., K. Ikuta, M. Naito, S. Ueda, S. Kato, and K. Hirai. 1987. Analysis of Marek's disease virus serotype 1-specific phosphorylated polypeptides in virus-infected cells and Marek's disease lymphoblastoid cells. J. Gen. Virol. **68**:1379-1389.

Nazerian, K., J. J. Solomon, R. L. Witter, and B. R. Burmester. 1968. Studies on the etiology of Marek's disease. II. Finding of a herpesvirus in cell culture. Proc. Soc. Exp. Biol. Med. **127**:177-182.

Nazerian, K. and R. L. Witter. 1970. Cell-free transmission and in vivo replication of Marek's disease virus. J. Virol. **5**:388-397.

Niedobitek, G., L. S. Young, R. Lau, L. Brooks, D. Greenspan, J. S. Greenspan, and A. B. Rickinson. 1991. Epstein-Barr virus infection in oral hairy leukoplakia: virus replication in the absence of a detectable latent phase. J. Gen. Virol. **72**:3035-3046.

Neipel, F., K. Ellinger, and B. Fleckenstein. 1991. The unique region of the human herpesvirus 6 genome is essentially collinear with the U_L segment of human cytomegalovirus. J. Gen. Virol. **72**:2293-2297.

Nicholas, J., K. R. Cameron, and R. W. Honess. 1992a. Herpesvirus saimiri encodes homologues of G protein-coupled receptors and cyclins. Nature **355**:362-365.

Nicholas, J., K. R. Cameron, H. Coleman, C. Newman, and R. W. Honess. 1992b. Analysis of nucleotide sequence of the rightmost 43 kbp of herpesvirus saimiri (HVS) L-DNA: general conservation of genomic organization between HVS and Epstein-Barr virus. Virology **188**:296-310.

Noble, G. A., G. T.-Y. Lee, R. Sprague, M. L. Parish, and P. G. Spear. 1983. Anti-gD monoclonal antibodies inhibit cell fusion induced by herpes simplex virus type 1. Virology **129**:218-224.

Nyaga, P. N. and D. G. McKercher. 1980. Pathogenesis of bovine herpesvirus-1 (BHV-1) infections: interactions of the virus with peripheral blood cellular components. Comp. Immun. Microbiol. Infect. Dis. **2**:587-602.

Okazaki, K., T. Matsuzaki, Y. Sugahara, J. Okada, M. Hasebe, Y. Iwamura, M. Ohnishi, T. Kanno, M. Shimizu, E. Honda, and Y. Kono. 1991. BHV-1 adsorption is mediated by the interaction of glycoprotein gIII with heparinlike moiety on the cell surface. Virology **181**:666-670.

Okazaki, W., H. G. Purchase, and B. R. Burmester. 1970. Protection against Marek's disease by vaccination with a herpesvirus of turkeys. Avian Dis. **14**:413-429.

Ono, K., S. Tanabe, M. Naito, T. Doi, and S. Kato. 1970. Antigen common to a herpes type virus from chickens with Marek's disease and EB virus from Burkitt's lymphoma cells. Biken's J. **13**:213-217.

Ozaki, T., T. Ichikawa, Y. Matsui, H. Kondo, T. Nagai, Y. Asano, K. Yamanishi, and M. Takahashi. 1986. Lymphocyte-associated viremia in varicella. J. Med. Virol. **19**:249-253.

Papavassiliou, A. G., K. W. Wilcox, and S. J. Silverstein. 1991. The interaction of ICP4 with cell/infected-cell factors and its state of phosphorylation modulate differential recognition of leader sequences in herpes simplex virus DNA. EMBO J. **10**:397-406.

Para, M. F., M. L. Parish, A. G. Noble, and P. G. Spear. 1985. Potent neutralizing activity associated with anti-glycoprotein D specificity among monoclonal antibodies selected for binding to herpes simplex virions. J. Virol. **55**:483-488.

Payne, L. N. 1982. Biology of Marek's disease virus and the herpesvirus of turkeys, p. 347-431. *In* B. Roizman (ed.), The herpesviruses, vol. 1. Plenum Publishing Corp., New York.

Peeters, B., N. de Wind, M. Hooisma, F. Wagenaar, A. Gielkens, and R. Moormann. 1992. Pseudorabies virus envelope glycoproteins gp50 and gII are essential for virus penetration, but only gII is involved in membrane fusion. J. Virol. **66**:894-905.

Pepose, J. S., J. G. Stevens, M. L. Cook, and P. W. Lampert. 1981. Marek's disease as a model for the Landry-Guillain-Barre Syndrome: latent viral infection in nonneuronal cells is accompanied by specific immune responses to peripheral nerve and myelin. Am. J. Pathol. **103**:309-320.

Pereira, M. S. 1972. Discussion summary, 497-498. *In* P. M. Biggs, G. de-The, and L. N. Payne (ed.), Oncogenesis and herpesviruses. IARC Scientific Publ. No. 2, Lyon, France.

Perry, L. and D. J. McGeoch. 1988. The DNA sequences of the long repeat region and adjoining parts of the long unique region in the genome of herpes simplex virus type 1. J. Gen Virol. 69:2831-2846.

Petrovskis, E. A., J. G. Timmins, M. A. Armentrout, C. C. Marchioli, R. J. Yancey, Jr., and L. E. Post. 1986a. DNA sequence of the gene for pseudorabies virus gp50, a glycoprotein without N-linked glycosylation. J. Virol. 59:216-223.

Petrovskis, E. A., J. G. Timmins, and L. E. Post. 1986b. Use of lambda gt11 to isolate genes for two pseudorabies virus glycoproteins with homology to herpes simplex virus and varicella-zoster virus glycoproteins. J. Virol. 60:185-193.

Petrovskis, E. A., and L. E. Post. 1987. A small open reading frame in pseudorabies virus and implications for evolutionary relationships between herpesviruses. Virology 159:193-195.

Petrovskis, E. A., A. L. Meyer, and L. E. Post. 1988. Reduced yield of infectious pseudorabies virus and herpes simplex virus from cell lines producing viral glycoprotein gp50. J. Virol. 62:2196-2199.

Plummer, G. 1967. Comparative virology of the herpes group. Progr. Med. Virol. 9:302-340.

Pol, J.M.A., F. Wagenaar, and A. Gielkens. 1991. Morphogenesis of three pseudorabies virus strains in porcine nasal mucosa. Intervirology. 32:327-337.

Post, L. E. and B. Roizman. 1981. A generalized technique for deletion of specific genes in large genomes: gene 22 of herpes simplex virus 1 is not essential for growth. Cell 25:227-232.

Powell, P. C. and J. G. Rowell. 1977. Dissociation of anti-viral and anti-tumor immunity in resistance to Marek's disease. J. Natl. Cancer Inst. 59:919-924

Purchase, H. G. and W. Okazaki. 1971. Effect of vaccination with herpesvirus of turkeys (HVT) on horizontal spread of Marek's disease. Avian Dis. 15:391-397.

Purves, F. C., M. Katan, W. S. Stevely, and D. P. Leader. 1986a. Characteristics of the induction of a new protein kinase in cells infected with herpesviruses. J. Gen. Virol. 67:1049-1057.

Purves, F. C., A. D. Deana, F. Marchiori, D. P. Leader, and L. A. Pinna. 1986b. The substrate specificity of the protein kinase induced in cells infected with herpesviruses: studies with synthetic substrates indicate structural requirements distinct from other protein kinases. Biochim. Biophys. Acta 889:208-215.

Purves, F. C., M. Katan, and D. P. Leader. 1987a. Complete purification of the pseudorabies virus protein kinase. Eur. J. Biochem. 167:507-512.

Purves, F. C., R. M. Longnecker, D. P. Leader, and B. Roizman. 1987b. Herpes simplex virus 1 protein kinase is encoded by open reading frame US3 which is not essential for virus growth in cell culture. J. Virol. 61:2896-2901.

Purves, F. C., D. Spector, and B. Roizman. 1991. The herpes simplex virus 1 protein kinase encoded by the U_S3 gene mediates posttranslational modification of the phosphoprotein encoded by the U_L34 gene. 1991. J. Virol. 65:5757-5764.

Qu, L. and D. T. Rowe. 1992. Epstein-Barr virus latent gene expression in uncultured peripheral blood lymphocytes. J. Virol. 66:3715-3724.

Quint, W., A. Gielkens, J. V. Oirschot, A. Berns, and H. T. Cuypers. 1987. Construction and characterization of deletion mutants of pseudorabies virus: a new generation of live vaccines. J. Gen. Virol. **68**:523-534.

Rall, G. F., Z. Lu, N. Sugg, R. A. Veach, and T. Ben-Porat. 1991. Acquisition of an additional cleavage site differentially affects the ability of pseudorabies virus to multiply in different host cells. J. Virol. **65**:6604-6611.

Rauh, I. and T. C. Mettenleiter. 1991. Pseudorabies virus glycoproteins gII and gp50 are essential for virus penetration. J. Virol. **65**:5348-5356.

Rea, T. J., J. G. Timmins, G. W. Long, and L. E. Post. 1985. Mapping and sequence of the gene for the pseudorabies virus glycoprotein which accumulates in the medium of infected cells. J. Virol. **54**:21-29.

Reilly, L. M., G. Rall, B. Lomniczi, T. C. Mettenleiter, S. Kupershmidt, and T. Ben-Porat. 1991. The ability of pseudorabies virus to grow in different hosts is affected by the duplication and translocation of sequences from the left end of the genome to the U_L-U_S junction. J. Virol. **65**:5839-5847.

Richman, D. D., A. Buckmaster, S. Bell, C. Hodgman, and A. C. Minson. 1986. Identification of a new glycoprotein of herpes simplex virus type 1 and genetic mapping of the gene that codes for it. J. Virol. **57**:647-655.

Rickinson, A. B., Q. Y. Yao, and L. E. Wallace. 1985. The Epstein-Barr virus as a model of virus-host interactions. Br. Med. Bull. **41**:75-79.

Riviere, M., J. Tartaglia, M. E. Perkus, E. K. Norton, C. Molnar Bongermino, F. Lacoste, C. Duret, P. Desmettre, and E. Paoletti. 1992. Protection of mice and swine from pseudorabies virus conferred by vaccinia virus-based recombinants. J. Virol. **66**:3424-3434.

Rixon, F. J. and J. B. Clements. 1982. Detailed structural analysis of two spliced HSV-1 immediate-early mRNAs. Nucl. Acids Res. **10**:2241-2256.

Rixon, F. J. and D. J. McGeoch. 1984. A 3' co-terminal family of mRNAs from the herpes simplex virus type 1 short region: two overlapping reading frames encode unrelated polypeptides one of which has a highly reiterated amino acid sequence. Nucl. Acids Res. **12**:2473-2487.

Rixon, F. J., C. Addison, and J. McLauchlan. 1992. Assembly of enveloped tegument structures (L particles) can occur independently of virion maturation in herpes simplex virus. J. Gen Virol. **73**:277-284.

Robertson, A. T., G. B. Caughman, W. L. Gray, R. P. Baumann, J. Staczek, and D. J. O'Callaghan. 1988. Analysis of the *in vitro* translation products of the equine herpesvirus type 1 immediate early mRNA. Virology **166**:451-462.

Roizman, B. 1972. The biochemical features of herpesvirus-infected cells, particularly as they relate to their potential oncogenicity- a review, p. 1-17. *In* P. M. Biggs, G. de-The, and L. N. Payne (eds.), Oncogenesis and herpesviruses. IARC Scientific Publications No. 2, Lyon, France.

Roizman, B., A. Bartha, P. M. Biggs, L. E. Carmichael, A. Granoff, P. Hampar, A. S. Kaplan, L. V. Melendez, K. Munk, A. Nahmias, G. Plummer, J. Rajcani, F. Rapp, M. Terni, G. de The, D. H. Watson, and P. Wildy. 1973. Provisional labels for herpesviruses. J. Gen. Virol. **20**:417-419.

Roizman, B., L. E. Carmichael, G. B. de-The, M. Masic, A. Nahmias, W. Plowright, F. Rapp, P. Sheldrick, M. Takashi, M. Terni, K. Wolfe. 1977. Provisional classification of herpesviruses, p. 1079-1082. *In* G. de-The, W. Henle, and F. Rapp (eds.), Oncogenesis and herpesviruses III, part 2. IARC Scientific Publications No. 24, Lyon, France.

Roizman, B., L. E. Carmichael, F. Deinhardt, G. de-The, A. J. Nahmias, W. Plowright, F. Rapp, P. Sheldrick, M. Takahashi, and K. Wolf. 1981. Herpesviridae: definition, provisional nomenclature, and taxonomy. Intervirology. **16**:201-217.

Roizman, B., Warren, J., C. A. Thuning, M. S. Fanshaw, B. Norrild, and B. Meignier. 1982. Application of molecular genetics to the design of live herpes simplex virus vaccines. Develp. Biol. Standard. 52:287-304.

Roizman, B., B. Norrild, C. Chan, and L. Pereira. 1984. Identification and preliminary mapping with monoclonal antibodies of a herpes simplex virus 2 glycoprotein lacking a known type 1 counterpart. Virology 133:242-247.

Roizman, B. 1990a. Whither herpesviruses?, p 285-291. *In* C. Lopez et al. (ed.), Immunobiology and prophylaxis of human herpesvirus infections. Plenum Press, New York.

Roizman, B. 1990b. Herpesviridae: a brief introduction, p. 1787-1793. *In* B. N. Fields et al. (ed.), Virology, 2nd ed., vol. 2. Raven Press, New York.

Roizman, B and A. E. Sears. 1990. Herpes simplex viruses and their replication, p. 1795-1841. *In* B. N. Fields et al. (ed.), Virology, 2nd ed., vol. 2. Raven Press, New York.

Roller, R. J. and B. Roizman. 1990. The herpes simplex virus Us11 open reading frame encodes a sequence-specific RNA-binding protein. J. Virol. 64:3463-3470.

Roller, R. J. and B. Roizman. 1991. Herpes simplex virus 1 RNA-binding protein Us11 negatively regulates the accumulation of a truncated viral mRNA. J. Virol. 65:5873-5879.

Roller, R. J. and B. Roizman. 1992. The herpes simplex virus 1 RNA binding protein Us11 is a virion component and associates with ribosomal 60S subunits. J. Virol. 66:3624-3632.

Ross, L.J.N., J. A. Frazier, and P. M. Biggs. 1972. An antigen common to some avian and mammalian herpesviruses, p.480-484. *In* P. M. Biggs, G. de-The, and L. N. Payne (ed.), Oncogenesis and herpesviruses. IARC Scientific Publ. No. 2, Lyon, France.

Ross, L.J.N., M. M. Binns, and J. Pastorek. 1991. DNA sequence and organization of genes in a 5.5 kbp Eco RI fragment mapping in the unique short segment of Marek's disease virus (strain RB1B). J Gen. Virol. 72:949-954.

Sacks, W. R. and P. A. Schaffer. 1987. Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICP0 exhibit impaired growth in cell culture. J. Virol. 61:829-839.

Salahuddin, S. Z., D. V. Ablashi, P. D. Markham, S. F. Josephs, S. Sturzenegger, M. Kaplan, G. Halligan, P. Biberfeld, F. Wong-Staal, B. Kramarsky, and R. C. Gallo. 1986. Isolation of a new virus, HBLV, in patients with lympholiferative disorders. Science 234:596-601.

Saltzman, R. L., M. R. Quirk, and M. Colin Jordan. 1988. Disseminated cytomegalovirus infection. Molecular analysis of virus and leukocyte interactions in viremia. J. Clin. Invest. 81:75-81.

Sanders, E.A.C.M., A.C.B. Peters, J. W. Gratana, and R.A.C. Hughes. 1987. GBS after varicella-zoster infection. Report of two cases. J. Neurol. 234:437-439.

Sanchez-Martinez, D., D. S. Schmid, W. Whittington, D. Brown, W. C. Reeves, S. Chatterjee, R. J. Whitley, and P. E. Pellett. 1991. Evaluation of a test based on baculovirus-expressed glycoprotein G for detection of herpes simplex virus type-specific antibodies. J. Infect. Dis. 164:1196-1199.

Schat, K. A., B. W. Calnek, J. Fabricant, and D. L. Graham. 1985. Pathogenesis of infection with attenuated Marek's disease virus strains. Avian Pathol. 14:127-146.

Schat, K. A., A. Buckmaster, and L.J.N. Ross. 1989. Partial transcriptional map of Marek's disease herpesvirus in lytically infected cells and lymphoblastoid cell lines. Int. J. Cancer. **44**:101-109.

Schat, K. A., C.-L.H. Chen, B. W. Calnek, and D. Char. 1991. Transformation of T-lymphocyte subsets by Marek's disease herpesvirus. J. Virol. **65**:1408-1413.

Schrier, R. D., J. A. Nelson, and M.B.A. Oldstone. 1985. Detection of human cytomegalovirus in peripheral blood lymphocytes in a natural infection. Science **230**:1048-1051.

Schultz, A. and S. Oroszlan. 1984. Myristilation of *gag-onc* fusion proteins in mammalian transforming retroviruses. Virology **133**:431-437.

Scott, J. C., S. K. Dutta, and A. C. Myrup. 1983. *In vivo* harboring of equine herpesvirus-1 in leukocyte populations and subpopulations and their quantitation from experimentally infected ponies. Am. J. Vet. Res. **44**:1344-1348.

Sears, A. E., I. W. Halliburton, B. Meignier, S. Silver, and B. Roizman. 1985. Herpes simplex virus 1 mutant deleted in the α22 gene: growth and gene expression in permissive and restricted cells and establishment of latency in mice. J. Virol. **55**:338-346.

Shiraki, K. and R. W. Hyman. 1987. The immediate early proteins of varicella-zoster virus. Virology **156**:423-426.

Silva, R. F. and R. L. Witter. 1985. Genomic expansion of Marek's disease virus DNA is associated with serial in vitro passage. J. Virol. **54**:690-696.

Sixbey, J. W., J. G. Nedrud, N. Raab-Traub, R. A. Hanes, and J. S. Pagano. 1984. Epstein-Barr virus replication in oropharyngeal epithelial cells. N. Engl. J. Med. **310**:1225-1230.

Smith, R. M., G. B. Caughman, and D. O'Callaghan. 1992. Characterization of the regulatory functions of the equine herpesvirus 1 immediate-early gene product. J. Virol. **66**:936-945.

Smith, T. 1934. *In* Parasitism and disease. Princeton Univ. Press, Princeton, New Jersey.

Spaete, R. R. and E. S. Mocarski. 1985. The a sequence of the cytomegalovirus genome functions as a cleavage/packaging signal for herpes simplex virus defective genomes. J. Virol. **54**:817-824.

Spear, P. G. 1984. Glycoproteins specified by herpes simplex viruses, p. 315-356. *In* B. Roizman (ed.), The herpesviruses, vol. 3. Plenum Press, New York.

Steiner, I., J. G. Spivack, R. P. Lirette, S. M. Brown, A. R. MacLean, J. H. Subak-Sharpe, and N. W. Fraser. 1989. Herpes simplex virus type 1 latency-associated transcripts are evidently not essential for latent infection. EMBO J. **8**:505-511.

Stenberg, R. M., A. S. Depto, J. Fortney, and J. A. Nelson. 1989. Regulated expression of early and late RNAs and proteins from the human cytomegalovirus immediate-early gene region. J. Virol. **63**:2699-2708.

Stevens, J. G. 1989. Human herpesviruses: a consideration of the latent state. Microbiol. Rev. **53**:318-332.

Su, H. K., R. Eberle, and R. J. Courtney. 1987. Processing of the herpes simplex virus type 2 glycoprotein gG-2 results in secretion of a 34,000-Mr cleavage product. J. Virol. **61**:1735-1737.

Sueoka, N. 1961. Variation and heterogeneity of base composition of deoxyribonucleic acids: a compilation of old and new data. J. Mol. Biol. **3**:31-40.

Sugaya, K., G. Bradley, M. Nonoyama, and A. Tanaka. Latent transcripts of Marek's disease virus are clustered in the short and long repeat regions. J. Virol. **64**:5773-5782.

Sugden, B. 1991. Herpes viruses: human transducing viruses. Trends Biochem. Sci. **16**:45-46.

Szilagyi, J. F. and C. Cunningham. 1991. Identification and characterization of a novel non-infectious herpes simplex virus-related particle. J. Gen. Virol. **72**:661-668.

Takahashi, K., S. Sonoda, K. Higashi, T. Kondo, H. Takahashi, M. Takahashi, and K. Yamanishi. 1989. Predominant CD4 T-lymphocyte tropism of human herpesvirus 6-related virus. J. Virol. **63**:3161-3163.

Takekoshi, M., S. Ihara, S. Tanaka, F. Maeda-Takekoshi, and Y. Watanabe. 1987. A new human cytomegalovirus isolate has an invertible subsegment within its L component producing eight genome isomers. J. Gen. Virol. **68**:765-776.

Telford, E.A.R., M. S. Watson, K. McBride, and A. J. Davison. 1992. The DNA sequence of equine herpesvirus-1. Virology 189:304-316.

Tham, K. M. and M. J. Studdert. 1987. Clinical and immunological responses of cats to feline herpesvirus 1 infection. Vet. Rec. **120**:321-326.

Thomsen, D. R., C. C. Marchioli, R. J. Yancey, Jr., and L. E. Post. 1987. Replication and virulence of pseudorabies virus mutants lacking glycoprotein gX. J. Virol. **61**:229-232.

Tikoo, S. K., D. R. Fitzpatrick, L. A. Babiuk, and T. J. Zamb. 1990. Molecular cloning, sequencing, and expression of functional bovine herpesvirus 1 glycoprotein gIV in transfected bovine cells. J. Virol. **64**:5132-5142.

Tomley, F., M. Binns, J. Campbell, and M. Boursnell. 1988. Sequence analysis of an 11.2 kilobase, near-terminal, BamHI fragment of fowlpox virus. J. Gen. Virol. **69**:1025-1040.

Towler, D. A., S. R. Eubanks, D. S. Towery, S. P. Adams, and L. Glaser. 1987. Amino-terminal processing of proteins by *N*-myristoylation. Substrate specificity of N-myristoyl transferase. J. Biol. Chem. **262**:1030-1036.

Umene, K. 1986. Conversion of a fraction of the unique sequence to part of the inverted repeats in the S component of the herpes simplex virus type 1 genome. J. Gen. Virol. **67**:1035-1048.

Umene, K. 1991. Recombination of the internal direct repeat element DR2 responsible for the fluidity of the a sequence of herpes simplex virus type 1. J. Virol. **65**:5410-5416.

Vafai, A., R. S. Murray, M. Wellish, M. Devlin, and D. H. Gilden. 1988a. Expression of varicella-zoster virus and herpes simplex virus in normal human trigeminal ganglia. Proc. Natl. Acad. Sci. USA **85**:2362-2366.

Vafai, A., Z. Wroblewska, R. Mahalingam, G. Cabirac, M. Wellish, M. Cisco, and D. Gilden. 1988b. Recognition of similar epitopes on varicella-zoster virus gpI and gpIV by monoclonal antibodies. J. Virol. **62**:2544-2551.

Vafai, A., K. Jensen, and R. Kubo. 1989. Existence of similar antigenic-sites on varicella-zoster virus gpI and gpIV. Virus Res. **13**:319-336.

van Zijl, M., H. van der Gulden, N. de Wind, A. Gielkens, and A. Berns. 1990. Identification of two genes in the unique short region of pseudorabies virus; comparison with herpes simplex virus and varicella-zoster virus. J. Gen Virol. **71**:1747-1755.

Varmuza, S. L. and J. R. Smiley. 1985. Signals for site-specific cleavage of HSV DNA: maturation involves two separate cleavage events at sites distal to teh recognition sequences. Cell **41**:793-802.

Wang, F.-I., V. F. Pang, and E. C. Hahn. 1988. Flow cytometric analysis of porcine peripheral blood leukocytes infected with pseudorabies virus. J. Leuk. Biol. **43**:256-264.

Watson, R. J., M. Sullivan, and G. F. Vande Woude. 1981. Structures of two spliced herpes simplex virus type 1 immediate-early mRNA's which map at the junctions of the unique and reiterated regions of the virus DNA S component. J. Virol. **37**:431-444.

Weber, P. C., M. Levine, and J. C. Glorioso. 1987. Rapid identification of nonessential genes of herpes simplex virus type 1 by Tn5 mutagenesis. Science **236**:576-579.

Weber, P. C., M. D. Challberg, N. J. Nelson, M. Levine, and J. C. Glorioso. 1988. Inversion events in the HSV-1 genome are directly mediated by the viral DNA replication machinery and lack sequence specificity. Cell **54**:369-381.

Weber, P. C., M. Levine, and J. C. Glorioso. 1990. Recombinogenic properties of herpes simplex virus type 1 DNA sequences resident in simian virus 40 minichromosomes. J. Virol. **64**:300-306.

Welch, H. M., C. G. Bridges, A. M. Lyon, L. Griffiths, and N. Edington. 1992. Latent equid herpesviruses 1 and 4: detection and distinction using the polymerase chain reaction and cocultivation from lymphoid cells. J. Gen. Virol. **73**:261-268.

Wen, L.-T., A. Tanaka, and M. Nonoyama. 1988. Identification of Marek's disease virus nuclear antigen in latently infected lymphoblastoid cells. J. Virol. **62**:3764-3771.

Weston, K. and B. G. Barrell. 1986. Sequence of the short unique region, short repeats and part of the long repeat of human cytomegalovirus. J. Mol. Biol. **192**:177-208.

Whealy, M. E., J. P. Card, R. P. Meade, A. K. Robbins, and L. W. Enquist. 1991. Effect of brefeldin A on alphaherpesvirus membrane protein glycosylation and virus egress. J. Virol. **65**:1066-1081.

Whittaker, G. R., L. A. Taylor, D. M. Elton, L. E. Giles, W. A. Bonass, I. A. Halliburton, R. A. Killington, and D. M. Meredith. 1992. Glycoprotein 60 of equine herpesvirus type 1 is a homologue of herpes simplex virus glycoprotein D and plays a major role in penetration of cells. J. Gen. Virol. **73**:801-809.

Whitton, J. L. and J. B. Clements. 1984a. The junctions between the repetitive and the short unique sequences of the herpes simplex virus genome are determined by the polypeptide-coding regions of two spliced immediate-early mRNAs. J. Gen. Virol. **65**:451-466.

Whitton, J. L. and J. B. Clements. 1984b. Replication origins and a sequence involved in coordinate induction of the immediate-early gene family are conserved in an intergenic region of herpes simplex virus. Nucl. Acids Res. **12**:2061-2069.

Wilcox, K. W., A. Kohn, E. Sklyanskaya, and B. Roizman. 1980. Herpes simplex virus phosphoproteins. I. Phosphate cycles on and off some viral polypeptides and can alter their affinity for DNA. J. Virol. **33**:167-182.

Wildy, P. 1973. Herpes: history and classification, p. 1-22. *In* A. S. Kaplan (ed.), The herpesviruses, Academic Press, New York and London.

Wirth, U. V., B. Vogt, and M. Schwyzer. 1991. The three major immediate-early transcripts of bovine herpesvirus 1 arise from two divergent and spliced transcription units. J. Virol. **65**:195-205.

Wirth, U. V., C. Fraefel, B. Vogt, C. Vlcek, V. Paces, and M. Schwyzer. 1992. Immediate-early RNA 2.9 and early RNA 2.6 of bovine herpesvirus 1 are 3' coterminal and encode a putative zinc finger transactivator protein. J. Virol. **66**:2764-2772.

Witter, R. L., J. J. Solomon, L. R. Champion, and K. Nazerian. 1971. Long-term studies of Marek's disease infection in individual chickens. Avian Dis. **15**:346-365.

Witter, R. L., J. M. Sharma, and A. M. Fadly. 1980. Pathogenicity of variant Marek's disease virus isolants in vaccinated and unvaccinated chickens. Avian Dis. **24**:210-232.

Witter, R. L. 1982. Protection by attenuated and polyvalent vaccines against highly virulent strains of Marek's disease virus. Avian Pathol. **11**:49-62.

Witter, R. L. 1983. Characteristics of Marek's disease viruses isolated from vaccinated commercial chicken flocks: association of viral pathotype with lymhoma frequency. Avian Dis. **27**:113-132.

Witter, R. L. 1987. New serotype 2 and attenuated serotype 1 Marek's disease viruses: comparative efficacy. Avian Dis. **31**:752-765.

Witter, R. L. 1991. Attenuated revertant serotype 1 Marek's disease viruses: safety and protective efficacy. Avian Dis. **35**:877-891.

Wittmann, G. and H.-J. Rziha. 1989. Aujeszky's disease (pseudorabies) in pigs, p. 230-333. *In* G. Wittmann (ed.), Herpesvirus diseases of cattle, horses and pigs, Kluwer Academic.

Wolf, K. and R. W. Darlington. 1971. Channel catfish virus: a new herpesvirus of ictalurid fish. J. Virol. **8**:525-533.

Yao, F. and R. J. Courtney. 1989. A major transcriptional protein (ICP4) of herpes simplex virus type 1 is associated with purified virions. J. Virol. **63**:3338-3344.

Yao, F. and R. J. Courtney. 1991. Association of a major transcriptional regulatory protein, ICP4, of herpes simplex virus type 1 with the plasma membrane of virus-infected cells. J. Virol. **65**:1516-1524.

Yalamanchili, R. R., B. Raengsakulrach, R. P. Baumann, and D. J. O'Callaghan. 1990. Identification of the site of recombination in the generation of the genome of DI particles of equine herpesvirus type 1. Virology **178**:448-455.

Yasukawa, M. and J. M. Zarling. 1985. Human cytotoxic T cell clones directed against herpes simplex virus-infected cells. III. Analysis of viral glycoproteins recognized by CTL clones by using recombinant herpes simplex viruses. J. Immunol. **134**:2679-2682.

Zhang, G., R. Stevens, and D. P. Leader. 1990. The protein kinase encoded in the short region of pseudorabies virus: description of the gene and identification of its product in virions and in infected cells. J. Gen. Virol. **71**:1757-1765.

Zhang, G. and D. P. Leader. 1990. The structure of the pseudorabies virus genome at the end of the inverted repeat sequences proximal to the junction with the short unique region. J. Gen. Virol. **71**:2433-2441.

Zsak, L., T. C. Mettenleiter, N. Sugg, and T. Ben-Porat. 1989. Release of pseudorabies virus from infected cells is controlled by several viral functions and is modulated by cellular components. J. Virol. **63**:5475-5477.

Zsak, L., F. Zuckermann, N. Sugg, and T. Ben-Porat. 1992. Glycoprotein gI of pseudorabies virus promotes cell fusion and virus spread via direct cell-to-cell transmission. J. Virol. **66**:2316-2325.

Zuckermann, F., T. Mettenleiter, C. Schreurs, N. Sugg, and T. Ben-Porat. 1988. Complex between glycoproteins gI and gp63 of pseudorabies virus: its effect on virus replication. J. Virol. **62**:4622-4626.

zur Hausen, H., H. Schulte-Holthausen, G. Klein, W. Henle, P. Clifford, and L. Santesson. 1970. EBV DNA in biopsies of Burkitt tumors and anaplastic carcinomas of the nasopharynx. Nature **228**:1056.

Chapter II

Structural characterization of the Marek's disease virus (MDV) unique short region: presence of alphaherpesvirus-homologous, fowlpox virus-homologous and MDV-specific genes

Submitted for publication.

ABSTRACT

Despite its classification as a gammaherpesvirus, primarily due to its lymphotropism, Marek's disease virus (MDV), an oncogenic avian herpesvirus, is phylogenetically more related to the "neurotropic" alphaherpesviruses, characterized by its prototype, herpes simplex virus (HSV) (Buckmaster et al., 1988, J. Gen Virol. 69:2033-2042). This raises interesting questions regarding the seeming incongruence between MDV's genetic- and biologic properties. Alphaherpesvirus U_S- and other S region genes originate from an area specific to members of this group, arguably their most divergent coding region. Moreover, this area contains a cluster of glycoprotein genes potentially important in protective immunity and possesses supplementary essential functions presumably evolved for adaptation to unique and diverse cell environments. In this report we present the nucleotide sequence of an 11,286 base pair DNA segment containing MDV's entire 11,160 bp long U_S region ("virulent" GA strain; vMDV). Eleven open reading frames (ORFs) likely to code for proteins were identified; of these, 7 represent homologs exclusive to alphaherpesvirus S component genes. These include MDV counterparts of HSV US1 (ICP22), US2, US3 (a serine-threonine protein kinase), US6, US7 and US8 (HSV glycoproteins gD, gI and gE, respectively) and US10. Three additional ORFs were identified with no apparent relation to any sequences currently present in the SwissProt or GenBank/EMBL databases, while a fourth was found to exhibit significant homology to an uncharacterized fowlpox virus (FPV) ORF. Having precisely identified the IR_S-U_S and U_S-TR_S junctions, we have corrected and clarified their previously reported locations. By characterizing genes encoding three new alphaherpesvirus-related homologs (US1, US8 and US10), completing the sequence for a fourth (US7) and identifying two new MDV-

specific ORFs (SORF1 and -3) and a fowlpox homolog (SORF2), our sequence analysis extends upon that of a 5,255-bp segment located in the U_S region of the "very virulent" RB1B strain of MDV (vvMDV) (Ross et al., 1991, J. Gen. Virol. 72:939-947; 949-954). The vMDV and vvMDV sequences above were found to be 99% identical at both nucleotide- and predicted amino acid levels. Combined with the fact that MDV U_S sequences failed to show statistically significant CpG deficiencies, our analysis is consistent with MDV bearing a closer phylogenetic relation to alphaherpesviruses. Inasmuch as the alphaherpesvirus-specific U_S region is known to specify a cluster of supplementary essential functions thought to be important in defining biological properties, our sequence provides a foundation for further MDV studies aimed at resolving the apparent discrepancy between MDV's genetic- and biologic properties.

INTRODUCTION

Marek's disease virus (MDV) is a highly pathogenic herpesvirus of chickens, which can cause; (i) T cell lymphomas as early as 3 weeks post-infection; (ii) peripheral neural lesions, characterized by lymphoproliferative infiltration and demyelination, occasionally leading to paralysis and/or blindness; (iii) various phenomena of acquired immnodeficiency and (iv) atherosclerosis in normocholesterolemic chickens, bearing a remarkable resemblance to the human disease, both in character and distribution of arterial lesions. Marek's disease (MD) is the clinical outcome of these various pathogenic manifestations (reviewed in Calnek & Witter, 1991) and was the first naturally occurring lymphomatous disorder to be effectively controlled by vaccination (Churchill et al., 1969).

Because of similar biological properties, especially its lymphotropism, MDV and its antigenically related, apathogenic vaccine virus, herpesvirus of turkeys (HVT) have been provisionally classified as gammaherpesviruses (Roizman et al., 1981). In contrast to gammaherpesviruses, MDV and HVT have genome structures more closely resembling those of alphaherpesviruses (Cebrian et al., 1982; Fukuchi et al., 1985; Igarashi et al., 1987). Consistent with their structural relatedness to alphaherpesviruses, recent data indicate that MDV and HVT are phylogenetically more related to alphaherpesviruses than gammaherpesviruses (Buckmaster et al., 1988). This raises interesting questions regarding the seeming incongruence between MDV's genetic- and biologic properties. To understand the nature of these differences, and to search for new glycoproteins potentially important in virus-host cell interactions, as well as the mechanism of protective immunity against MD, we have become particularly interested in the MDV U_S region. This stems from the observation that

alphaherpesvirus U_S regions are known to contain a cluster of glycoprotein genes and appear to specify determinants for pathogenesis and viral dissemination, rather than those essential for virus production (Roizman, 1990a). These determinants are encoded by a cluster of "non-essential"- or *supplementary essential* genes which are likely to account for many of the unique in vivo properties characteristic for a given alphaherpesvirus. The natural host MDV system affords a unique opportunity to examine the *in vivo* role and function of this putative class of supplementary essential genes.

The alphaherpesvirus U_S region is flanked by a pair of inverted repeat sequences (inverted- and terminal repeat short, IR_S and TR_S, respectively, or simply, repeat short, R_S). Together, these components make up the S region. Alphaherpesvirus S regions are distinguished by marked differences in content, genetic organization and evolutionary divergence (e.g. HSV-1 U_S = 13.0 kbp, 12 genes (McGeoch et al., 1985); varicella-zoster virus (VZV) U_S = 5.2 kbp, 4 genes (Davison & Scott, 1986). Secondly, they specify a cluster of glycoprotein genes potentially important for protective immunity. A similar cluster in MDV would be particularly significant given the paucity of specific details regarding protective immunity against naturally occurring Marek's disease (MD) tumors.

The DNA sequence of a 5,255-bp segment from U_S region of the "very virulent" RB1B strain of MDV (vvMDV) was recently reported (Ross et al., 1991). This region was found to contain open reading frames (ORFs) homologous to proteins encoded by HSV US2, US3 (protein kinase), US6 (glycoprotein D), part of US7 (glycoprotein I), and an additional MDV-specific ORF. In this report, we extend upon these results and present a sequence analysis of the entire 11.2 kbp MDV U_S region ("virulent" GA strain, vMDV). Compared with the 5.3 kbp RB1B segment of MDV, the corresponding GA sequence was 99% identical at both nucleotide- and predicted amino acid levels. In addition to the

alphaherpesvirus U_S homologs shared in common with the RB1B strain, we have completed the US7 (gI) sequence and have identified three additional ORFs homologous to HSV US1 (ICP22), -US8 (gE), and -US10; a fowlpox virus (FPV) homolog; and at least two additional MDV-specific ORFs.

MATERIALS AND METHODS

Recombinant plasmids, M13 subcloning and DNA sequencing. Pathogenic MDV GA strain subclones included EcoRI-O, -I and -V cloned into pBR328 (Gibbs et al., 1984) (pE328-O, pE328-I, and pE328-V, Figure 1B); BamHI-A and BamHI-P1, cloned into pACYC184 and pBR322, respectively (Fukuchi et al., 1985) (pBACYC-A and pB322-P1 (Figure 1B), kindly provided by Dr. Meihan Nonoyama of the Tampa Bay Research Institute, St. Petersburg, FL); and GA-02, a phage clone containing a partially digested MDV Sau3A insert cloned into the SalI site of EMBL3, kindly provided by Dr. Paul J. A. Sondermeijer, Intervet International, Boxmeer, The Netherlands. The latter clone contains most of BamHI-A, all of BamHI-P1 and additional 3'-flanking sequences, including some of those present in pE328-V. This phage clone was used to generate the pUC18 subclone, pSP18-A (Figure 1B). This clone contains a 2.5 kb SalI insert with approximately 20 bp of EMBL-3's multiple cloning site at its 3' end. Together, the above clones (Figure 1B) were used to generate M13mp18 and -19 subclones for use as templates for nucleotide sequencing.

DNA sequencing of both strands was performed by the dideoxy-chain termination method (Sanger et al., 1977) using single-stranded M13 templates. Reaction products were synthesized and labeled using a 17-mer M13 primer, a modified T7 DNA polymerase (Sequenase), [^{35}S] thio-dATP (NEN) and appropriate deoxy- and dideoxynucleotides according to instructions by the manufacturer (Sequenase sequencing kit; United States Biochemical Corp., Cleveland, Ohio) and electrophoresed through 7% polyacrylamide/50% urea/Tris-Borate-EDTA gels. Remaining sequence gaps were determined by substituting M13 primers with synthetic 17-mer oligonucleotides (under similar

reaction conditions, 0.5 pmoles/reaction) generated based on previously determined sequences.

Analysis of sequence data. Sequences were assembled and analyzed with an IBM Personal System 2/Model 50 microcomputer utilizing Genepro (Version 4.10; Riverside Scientific Enterprises, Seattle, WA) sequence analysis software packages or programs obtained from the University of Wisconsin Genetics Computer Group (UWGCG, Versions 6.2 and 7.0; Devereaux et al., 1984) and run through a VAX 8650 minicomputer. Homology searches of the SwissProt (Release 18.0, 5/91), GenBank (Release 71.0, 3/92) and EMBL (Release 30.0, 3/92) databases were performed using the UWGCG programs FASTA and TFASTA (Pearson & Lipman, 1988). FASTA was utilized against both protein (SwissProt) and nucleic acid (GenBank/EMBL) databases, while TFASTA was used to compare protein sequences against GenBank/EMBL. Briefly, these programs employ an algorithm to locate regions of similarity, a PAM250-based scoring system to provide a qualitative and quantitative homology assessment and an alignment procedure to join together, when possible, the highest-scoring, non-overlapping regions in order to derive an alignment and its resulting, optimized score. Dot matrix homology plots were generated using the UWGCG program DOTPLOT with the output file from UWGCG's COMPARE. To create multiple alignments, successive GAP comparisons were conducted between MDV and its homologous sequences (in descending order of homology), generating gapped output files to be used as input sequences for subsequent runs of GAP until the alignment of these gapped sequences could no longer be expanded by the addition of new gaps. Following alignment, the gapped output files were displayed and a consensus sequence calculated using the UWGCG program, Pretty. For optimal results, manual editing, based on visual inpection, was employed (using UWGCG's LINEUP). When using FASTA, TFASTA,

COMPARE and GAP, "similar" amino acids were defined as amino acid comparisons equal to, or exceeding +0.5, as derived from Dayhoff's mutational matrix table by rescaling the values by dividing each by the sum of its row and column, and normalizing to a mean of 0 and standard deviation of 1.0, where perfect matches are set to +1.5; these and other values ranging between -1.1 and +1.5 are described in the UWGCG User's Guide appendix or accessible by typing the command FETCH, followed by ComparPep.Cmp or NWSGapPep.Cmp.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned the accession number XNNNNN.

RESULTS

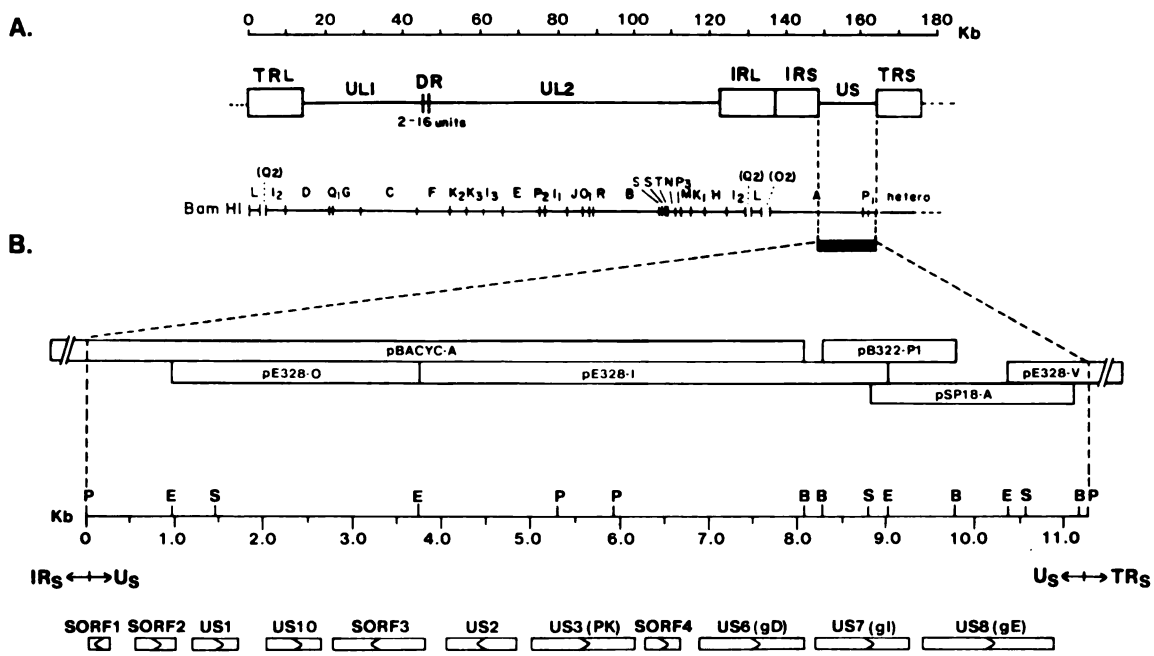
Defining the MDV U_S region and the location of the unique-repeat region junctions. Figure 1 contains a map of the area that was sequenced. This segment is bounded by a pair of PvuII sites and spans the 3' half of BamHI-A, extending an additional 1.5 kbp to the right of the 3' end of BamHI-P1 (Figs. 1B and 2). This 11,286-bp segment spans the entire U_S region, which is 11,160 bp in length and flanked at the 5' and 3' ends by a 63 bp stretch of IR_S and TR_S DNA, respectively, each inversely complementary to the other (Figs. 1B and 2). Based on Southern blot analysis, the IR_S-U_S junction was previously localized to a 1.4 kb Bgl I fragment (Fukuchi et al., 1985) located in the second of five EcoRI subfragments of BamHI-A (for BamHI-A/EcoRI map see Wen et al., 1988). Our sequence analysis provides conclusive proof that this junction is actually located in the middle of the third EcoRI subfragment, approximately 2-3 kbp downstream from the reported position. This is further supported by our own Southern blot analysis (data not shown). The U_S/TR_S junction is located 263 nucleotides downstream of the US8 termination codon (following position 11,223, Figure 2). Identifying the MDV U_S/TR_S junction necessitated the characterization of previously unreported clones mapping 3' of BamHI-P1, extending into the TR_S region. Assuming that the MDV IR_S is identical to its TR_S, the availability of these new clones should now allow for the complete characterization of MDV's S region.

Nucleotide sequence and identification of open reading frames. The overall guanine plus cytosine ratio of the region sequenced was found to be 41%, somewhat below the reported genomic value of 46% (Calnek & Witter, 1991). Observed frequencies of CpG dinucleotides in the whole sequence, or in the coding regions only, did not differ significantly from those expected from

FIGURE 1. Map location of area sequenced and organization of MDV U_S ORFs.

A) MDV genome structure and BamHI restriction map outlining area sequenced. Boxes define plasmid clones with BamHI, EcoRI or SalI-bound inserts that were used to generate M13mp18 and -19 templates for sequencing.

B) Organization of MDV U_S ORFs. Boxes represent location of MDV ORFs. Arrows define direction of transcription/translation. Names of ORFs are displayed above boxes. Basis for nomenclature is outlined in RESULTS.


A.
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ULI
DR
2-16 units
UL2
IRL
IRS
US
TRS
Bam HI
B.
pBACYC·A
pB322·P1
pE328·O
pE328·I
pE328·V
pSP18·A
Kb
0 1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0 11.0
IRS←→US
US←→TRS
SORF1 SORF2 US1 US10 SORF3 US2 US3(PK) SORF4 US6(gD) US7(gI) US8(gE)

their mononucleotide compositions (data not shown). This result agrees with those obtained from alphaherpesviruses, while sharply contrasting with those obtained from all gammaherpesviruses thus far studied (Efstathiou et al., 1990; Honess et al., 1989), including the A+T rich herpesvirus saimiri (HVS) and the G+C rich Epstein-Barr virus (EBV), which are both deficient in CpG dinucleotides.

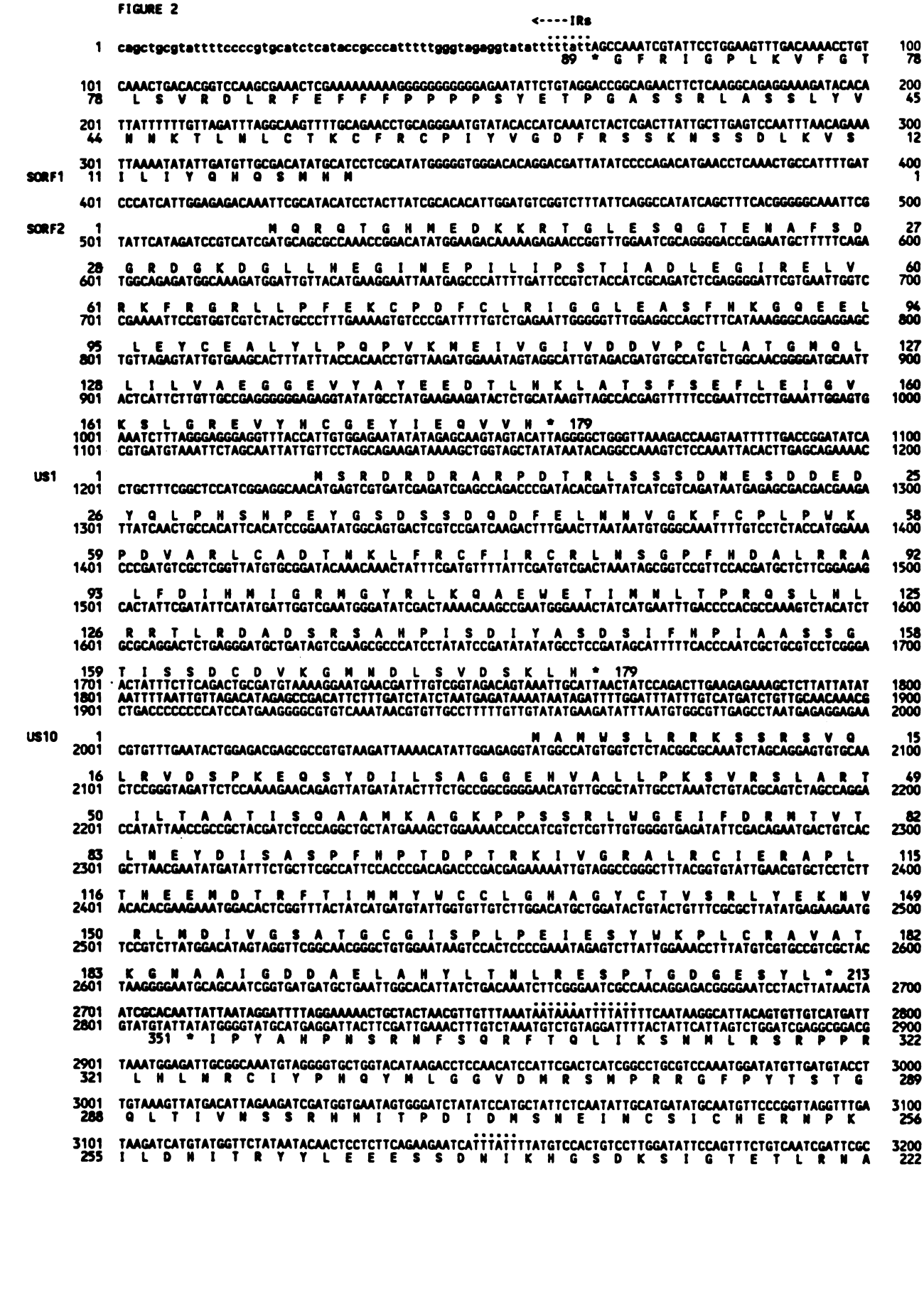
The region sequenced contains at least 11 ORFs likely to code for proteins (Figure 1B; basis for names is defined below). This prediction was primarily based on homology and positional organization comparisons to other alphaherpesvirus genes. This identification was further guided by the observation that alphaherpesviruses such as HSV and VZV tend to contain relatively tightly packed, unspliced coding regions (Davison & Scott, 1986; McGeoch et al., 1985, 1987, 1988). Methods for detecting protein coding regions based on the use of MDV-derived codon frequency tables (using these and previously published MDV sequences; Binns and Ross, 1989; Ross et al., 1989; Scott et al., 1989) or analysis of compositional bias (using the UWGCG programs CODONPREFERENCE and TESTCODE) were inconclusive. However, as pointed out previously (Ross et al., 1991), MDV does appear to contain a detectable bias for A-T residues in the wobble position. Furthermore, using the UWGCG program FRAMES, together with the MDV-derived codon frequency table above, the 11 identified ORFs clearly show a significantly low pattern of rare codon usage not observed following computer-based translation of the remaining reading frames (data not shown).

The predicted amino acid sequences of the putative ORFs (beginning from the first ATG codon) are shown relative to the nucleotide sequence in Figure 2. Due to the A-T rich nature of MDV, there are numerous TATA-like sequences for transcriptional initiation, more than are likely to have functional

FIGURE 2. Nucleotide and predicted amino acid sequences.

The nucleotide sequence is given as the rightward 5' to 3' strand only (numbered 1 to 11,286). IR_S and TR_S sequences are located at the 5' and 3' ends, respectively, and are depicted using lower case symbols; U_S sequences are in upper case. Rightward- and leftward-directed predicted amino acid sequences are shown above and below the corresponding nucleotide sequences, respectively, in single-letter code. The name of each ORF is given to the left of the first line of its respective sequence. Amino acid sequences are numbered from the N terminus, beginning with the first in-frame methionine codon and ending with the amino acid at the C-terminus, which precedes the termination codon. Dotted lines identify potential polyadenylation signals. Putative signal peptide and transmembrane domain regions of MDV US6 (gD), -US7 (gI), and -US8 (gE) are overlined at the amino- and carboxy ends, respectively. Signal peptide overlining continues through to the last amino acid to the left of the predicted cleavage site (von Heijne, 1986). Potential N-glycosylation sites (N-X-S/T) are indicated by dashed lines.

FIGURE 2



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        3401 TTCCTATGGAAAGCATTGTGGTCCAGTTTTCCAGATGAAATGAAAACAATGCGGGCAAAAATGGTCCCACCTGTTTCATCTTCAATGCATCTCTCACATC 3500
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         88   F F R Y A S W I V M Q C W M D K H I D F S T I S G E N T S I T C L  56

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SORF3    21   K G F R G R P I D M D D F M T A N V R S M  1

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        4001 GGTTGTAGCGATGCACCTAATCGTAATGTGTATACGCCCCATCATGTAATTATATCTAATTGGTAGCAAGTAGGTCTGTCGAATAACAGCTAATGACTAC 4100
                                                                                270 * N S  269

        4101 CGGCTCTACATTTTTTCTGTATTCGTGACTTTCCTGTCGCAGTGTAACGAACCGGAATTGCAATCGCATCTCTATCTTCTTTCTTGCAACATTTTCCACA 4200
         268   G A R C K K Q I R S K G T A T Y R V P I A I A D R D E K K C C K G C  235

        4201 ACAGAATAATCTGCCGGGTGTACTACTCATTTGAGGTGGTTCGATTTCCGGAGGTTTTAGAGGATTGGGTGGGGACCCGAGGATTTTGTATACACATACC 4300
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        4401 GCATACCGGACCAGGTCCCAGACACTTTGATTGCAAGTAACCTTTTTGGCAAAGGAATACATTCGAGCGCAATGGCACATATATCTGCCGCCCCAACTAT 4500
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US2      1   M
US3      1                 M S S T P E A E T M E C G I S S S K V H D S K T M T T Y G  29
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         264   P E L L A L D P Y C T K T D I W S A G L V L F E M S V K N I T F F  296
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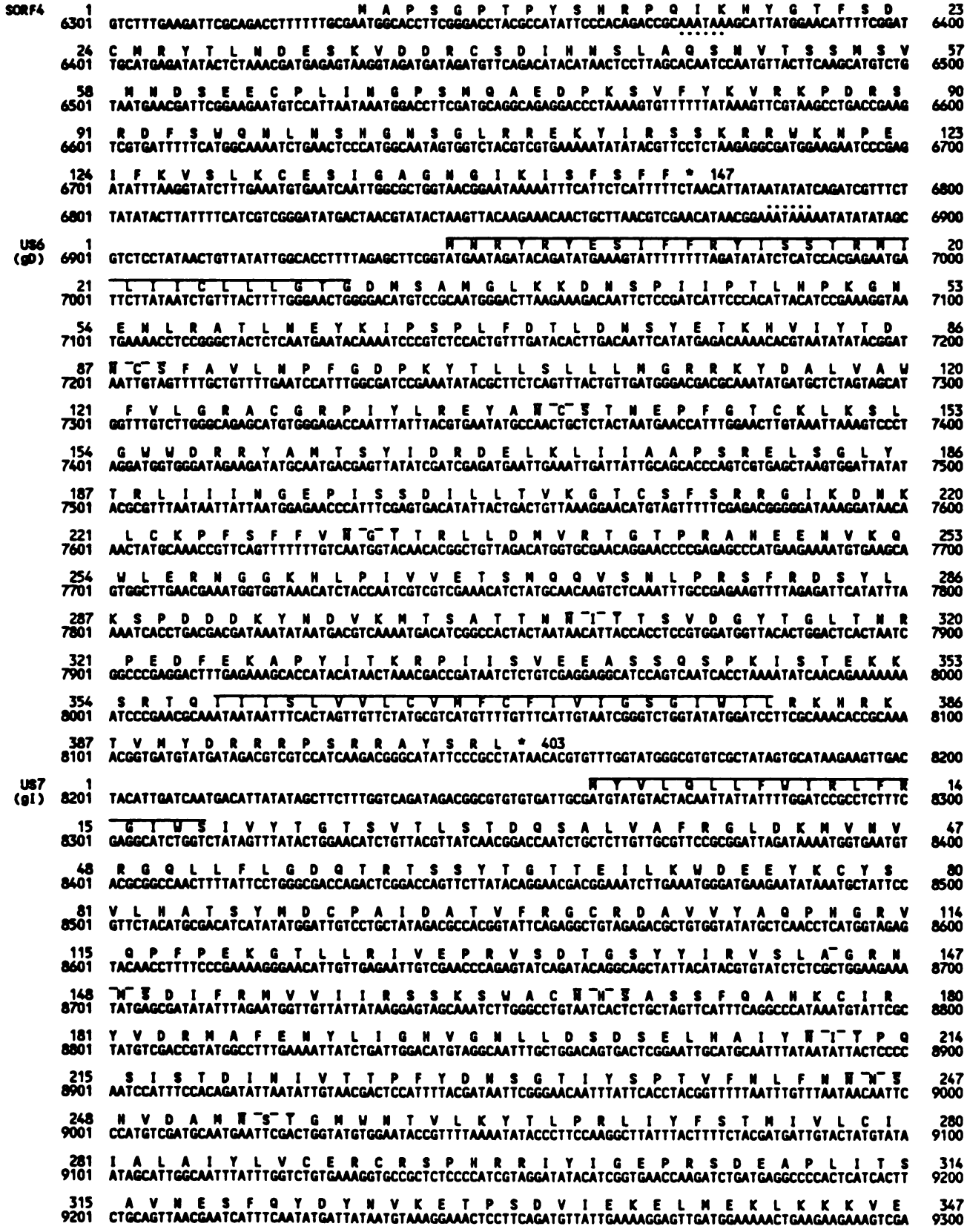

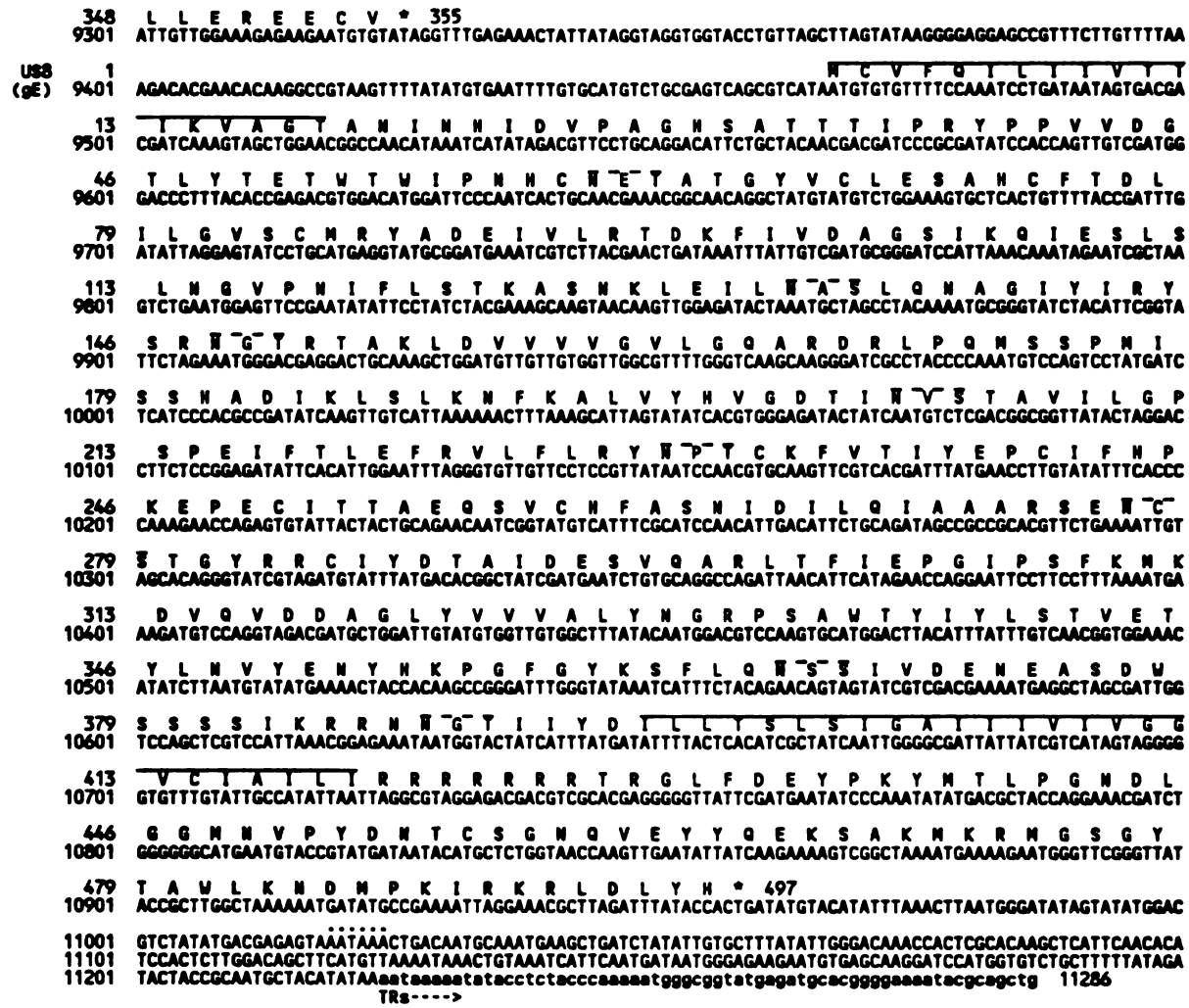
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US8 1 M C V F Q I L I I V T T 12
(gE) 9401 AGACACGAACACAAGGCCGTAAGTTTTATATGTGAATTTTGTGCATGTCTGCGAGTCAGCGTCATAATGTGTGTTTTCCAAATCCTGATAATAGTGACGA 9500
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179 S S N A D I K L S L K N F K A L V Y H V G D T I N V S T A V I L G P 212
10001 TCATCCCACGCCGATATCAAGTTGTCATTAAAAAACTTTAAAGCATTAGTATATCACGTGGGAGATACTATCAATGTCTCGACGGCGGTTATACTAGGAC 10100
213 S P E I F T L E F R V L F L R Y N P T C K F V T I Y E P C I F N P 245
10101 CTTCTCCGGAGATATTCACATTGGAATTTAGGGTGTTGTTCCTCCGTTATAATCCAACGTGCAAGTTCGTCACGATTTATGAACCTTGTATATTTCACCC 10200
246 K E P E C I T T A E Q S V C N F A S N I D I L Q I A A A R S E N C 278
10201 CAAAGAACCAGAGTGTATTACTACTGCAGAACAATCGGTATGTCATTTCGCATCCAACATTGACATTCTGCAGATAGCCGCCGCACGTTCTGAAAATTGT 10300
279 S T G Y R R C I Y D T A I D E S V Q A R L T F I E P G I P S F K N K 312
10301 AGCACAGGGTATCGTAGATGTATTTATGACACGGCTATCGATGAATCTGTGCAGGCCAGATTAACATTCATAGAACCAGGAATTCCTTCCTTTAAAATGA 10400
313 D V Q V D D A G L Y V V V A L Y N G R P S A W T Y I Y L S T V E T 345
10401 AAGATGTCCAGGTAGACGATGCTGGATTGTATGTGGTTGTGGCTTTATACAATGGACGTCCAAGTGCATGGACTTACATTTATTTGTCAACGGTGGAAAC 10500
346 Y L N V Y E N Y H K P G F G Y K S F L Q N S T S I V D E N E A S D W 378
10501 ATATCTTAATGTATATGAAAACTACCACAAGCCGGGATTTGGGTATAAATCATTTCTACAGAACAGTAGTATCGTCGACGAAAATGAGGCTAGCGATTGG 10600
379 S S S S I K R R N N G T I I Y D I L L I S L S I G A I I I V I V G G 412
10601 TCCAGCTCGTCCATTAAACGGAGAAATAATGGTACTATCATTTATGATATTTTACTCACATCGCTATCAATTGGGGCGATTATTATCGTCATAGTAGGGG 10700
413 V C I A I L I R R R R R R R T R G L F D E Y P K Y M T L P G N D L 445
10701 GTGTTTGTATTGCCATATTAATTAGGCGTAGGAGACGACGTCGCACGAGGGGGTTATTCGATGAATATCCCAAATATATGACGCTACCAGGAAACGATCT 10800
446 G G N N V P Y D N T C S G N Q V E Y Y Q E K S A K M K R M G S G Y 478
10801 GGGGGGCATGAATGTACCGTATGATAATACATGCTCTGGTAACCAAGTTGAATATTATCAAGAAAAGTCGGCTAAAATGAAAAGAATGGGTTCGGGTTAT 10900
479 T A W L K N D M P K I R K R L D L Y H * 497
10901 ACCGCTTGGCTAAAAAATGATATGCCGAAAATTAGGAAACGCTTAGATTTATACCACTGATATGTACATATTTAAACTTAATGGGATATAGTATATGGAC 11000
11001 GTCTATATGACGAGAGTAAATAAACTGACAATGCAAATGAAGCTGATCTATATTGTGCTTTATATTGGGACAAACCACTCGCACAAGCTCATTCAACACA 11100
11101 TCCACTCTTGGACAGCTTCATGTTAAAATAAACTGTAAATCATTCAATGATAATGGGAGAAGAATGTGAGCAAGGATCCATGGTGTCTGCTTTTTATAGA 11200
11201 TACTACCGCAATGCTACATATAAaaataaaaatatacctctacccaaaaatgggcggtatgagatgcacgggggaaaatacgcagctg 11286
TRs---->

relevance. Therefore, these sites have not been highlighted in Figure 2. Proposed ORF and predicted polyadenylation signal locations, identification of the -3, +4 translational context nucleotides (Kozak, 1989), as well as the lengths, predicted molecular masses and predicted isoelectric points of the predicted translational products are shown in Table 1. Predicted polyadenylation site locations are based on the pattern of HSV1, in which U_S genes have been found to share these signals, utilizing the nearest available site located within or near intergenic regions, resulting in the transcription of 3' coterminal mRNA families (McGeoch et al., 1985; Rixon & McGeoch, 1985). Because these ORFs have not yet been characterized and to simplify identification, seven have been named (Figure 1B, Table 1) based on homology (see below) to HSV-1-encoded U_S ORFs (McGeoch et al., 1985). When appropriate, the letters MDV will preface the homolog's name to indicate the ORF's origin. The four non-alphaherpesvirus related ORFs have been arbitrarily named SORFs 1, -2, -3 and -4 (unique short region open reading frame).

According to the scanning model for translation, the 40S ribosomal subunit binds initially at the 5'-end of mRNA and then migrates, stopping at the first AUG (ATG) codon in a favorable context for initiating translation (reviewed in Kozak, 1989). As long as there is a purine in position -3, deviations from the rest of the consensus only marginally impair initiation (Kozak, 1989). In the absence of such a purine, however, a guanine at position +4 is essential for efficient translation. In the absence of S1 nuclease and/or primer extension analysis, definitive start sites for translation cannot be predicted with any certainty. Except for SORF2 and -4, all of the ORFs contain a potential initiation codon with a purine residue in the -3 position. In the case of these two ORFs, however, a compensating guanine in the +4 position corresponding to an

TABLE 1. SUMMARY OF MDV U_S ORF DATA

Name	ORF Start	ORF Stop	Predicted Polyadenylation Site	-3, +4[a] Context Nucleotides	Codons	Mr[b]	pI[c]
SORF1	331	62	63	A,C	89	10.1	10.3
SORF2	521	1060	2756	T,C; C,G	179	20.1	4.5
US1	1227	1766	2756	A,A	179	20.4	6.5
US10	2056	2697	2756	G,G	213	23.6	8.2
SORF3	3863	2808	2769	A,A	351	40.6	8.2
US2	4902	4093	2769	A,G	270	29.7	7.6
US3	5014	6222	6372	T,T; A,G	402	44.7	6.1
SORF4	6332	6775	6883	C,G	147	16.8	9.8
US6	6943	8154	11019	G,G	403	42.6[d]	10.3[d]
US7	8261	9328	11019	G,T	355	38.3[d]	6.7[d]
US8	9467	10960	11019	A,T	497	53.7[d]	8.0[d]

[a]Numbering begins with A of ATG (AUG) codon as position +1; nucleotides 5' to that site are assigned negative numbers.

[b]In absence of post-translational modifications.

[c]Calculated using the UWGCG program, ISOELECTRIC.

[d]Based on sequences that follow the predicted signal peptide cleavage site.

alternative, downstream initiation codon located a short distance away (Table 1).

Database- and computer-assisted homology comparisons. Using the computer program FASTA or TFASTA (Pearson & Lipman, 1988), each of the 11 predicted amino acid sequences was screened against the SwissProt protein database or GenBank/EMBL nucleic acid databases, respectively, in addition to recently published pseudorabies virus (PRV) (van Zijl et al., 1990) and equine herpesvirus-1 (EHV-1) (Colle et al., 1992) S segment gene sequences not present in these databases. Optimized FASTA/TFASTA scores greater than 100 were initially considered as potential candidates possessing a significant degree of amino acid similarity. The results of this analysis are in Table 2. Apart from MDV US3, six ORFs (MDV US1, -10, -2, -6, -7 and -8; Tables 1, 2) were found to be exclusively homologous to alphaherpesvirus S segment genes (Table 2); in contrast, SORF1, -3 and -4 failed to show statistically significant homology with any sequences in either of the two databases. On the other hand, using SORF2 as a probe for FASTA analysis, a FASTA score of 237 was obtained, indicating homology to an uncharacterized fowlpox virus (FPV) ORF (e.g. FPV ORF4; Tomley et al., 1988). Upon alignment, these sequences were found to exhibit 67% similarity and 42% identity over the 100 aa aligned (Figure 3). Like other US3 homologs, MDV's counterpart exhibits homology to the serine-threonine protein kinase superfamily (Hanks et al., 1988), as evidenced by a relatively large number of FASTA scores between 150 and 250. Nevertheless, these scores were 3-4 fold lower than those obtained between US3 homologs of HSV, VZV and PRV (Table 2). The US3 gene family of herpesvirus protein kinases appear to define a distinct subfamily within the serine-threonine protein kinase superfamily; it is thought that related cellular counterparts exist and await future characterization (Hanks et al., 1988). Analyses of homologies to HSV-2 US2, -3,

TABLE 2. PAIRWISE COMPARISONS OF MDV AND ALPHAHERPESVIRUS S REGION HOMOLOGS

		US1					US10				US2			US3			
	Virus	MDV	HSV-1	VZV	PRV	EHV-4	MDV	HSV-1	VZV	EHV-4	MDV	HSV-1	PRV	MDV	HSV-1	VZV	PRV
% similar / %identical	MDV	-	47/26	43/27	51/33	48/30	-	45/24	40/24	45/29	-	51/33	48/26	-	56/38	54/33	55/33
	HSV-1	47/26	-	49/29	43/25	50/29	45/24	-	49/27	49/27	51/33	-	50/31	56/38	-	57/41	59/36
	VZV	43/27	49/29	-	51/35	54/36	40/24	49/27	-	55/32	a	a	a	54/33	57/41	-	58/35
	PRV	51/33	43/25	51/35	-	56/41	a	a	a	a	48/26	50/31	-	55/33	59/36	58/35	-
	EHV-4	48/30	50/29	54/36	56/41	-	45/29	49/27	55/32	-	a	a	a	a	a	a	a
FASTA scores	MDV	891	101	160	218	208	1071	134	147	251	1421	335	**118	1931	611	616	563
	HSV-1	101	2047	119	201	150	134	1617	123	180	335	1554	112	611	2409	717	620
	VZV	160	119	1378	340	359	147	123	978	191	a	a	a	616	717	1960	595
	PRV	218	201	340	1724	525	a	a	a	a	**168	112	1240	563	620	595	1948
	EHV-4	208	150	359	525	1308	251	180	191	1312	a	a	a	a	a	a	a
	length (aa)	179	420	278	364	*273	213	312	180	259	270	291	256	390	481	393	390

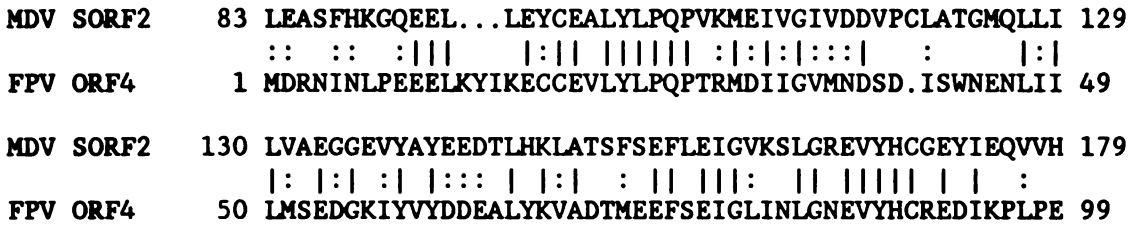
		US6					US7					US8				
	Virus	MDV	HSV-1	PRV	EHV-1	BHV-1	MDV	HSV-1	VZV	PRV	EHV-1	MDV	HSV-1	VZV	PRV	EHV-1
% similar / %identical	MDV	-	42/21	44/23	43/21	42/33	-	39/22	46/23	43/25	41/23	-	44/22	43/22	46/28	47/22
	HSV-1	42/21	-	47/27	44/22	50/28	39/22	-	43/24	41/26	42/23	44/22	-	46/27	49/28	41/23
	VZV	b	b	b	b	b	46/23	43/24	-	47/25	46/29	43/22	46/27	-	47/25	46/29
	PRV	44/23	47/27	-	51/30	57/38	43/25	41/26	47/25	-	51/30	46/28	49/28	49/29	-	54/34
	EHV-1	43/21	44/22	51/30	-	52/30	41/23	42/23	46/29	51/30	-	47/22	41/23	50/28	54/34	-
	BHV-1	42/33	50/28	57/38	52/30	-	a	a	a	a	a	a	a	a	a	a
FASTA scores	MDV	2068	211	279	246	291	1816	145	228	184	242	2489	192	376	**243	399
	HSV-1	211	1999	294	253	304	145	1880	234	188	249	192	2751	357	257	274
	VZV	b	b	b	b	b	228	234	1705	198	298	376	357	3171	329	468
	PRV	279	294	2116	428	730	188	188	198	1652	274	**217	257	329	2923	417
	EHV-1	246	253	428	1995	494	242	249	298	274	1979	399	274	468	417	2821
	BHV-1	291	304	730	494	2148	a	a	a	a	a	a	a	a	a	a
	length (aa)	403	394	402	395	417	355	390	354	350	424	497	550	623	577	552

a existence of homolog undetermined
b no homolog present in genome
* actual length will differ somewhat, since probable initiation codon not defined
** different score when order of comparison reversed

FIGURE 3. Homology between MDV SORF2 and FPV ORF4.

GAP (UWGCG) analysis aligning area conserved between SORF2 and fowlpox virus ORF4 (Tomley et al., 1988). Amino acid numbers (with respect to predicted 5' ATG) of aligned sequences are listed at the beginning and end of each line. Bars and double dots identify identical and similar amino acid matches, respectively. The area aligned was 67% similar, 42% identical; a FASTA score of 237 was obtained.

FIGURE 3


MDV SORF2 83 LEASFHKGQEEL...LEYCEALYLPQPVKMEIVGIVDDVPCLATGMQLLI 129
FPV ORF4 1 MDRNINLPEEELKYIKECCEVLYLPQPTRMDIIGVMNDSD.ISWNENLII 49
MDV SORF2 130 LVAEGGEVYAYEEDTLHKLATSFSEFLEIGVKSLGREVYHCGEYIEQVVH 179
FPV ORF4 50 LMSEDGKIYVYDDEALYKVADTMEEFSEIGLINLGNEVYHCREDIKPLPE 99

-6 and -7 are not presented in this report, inasmuch as their ORFs exhibit greater than 70% identity to their HSV-1 counterparts (McGeoch et al., 1987) and result in homologies with MDV that basically resemble those with HSV-1. MDV US6 exhibits demonstrable homology to HSV-2 US4 (FASTA=100) and its PRV counterpart, gX (FASTA=90). This is consistent with earlier findings suggesting duplication and divergence of S component glycoprotein genes from common precursors (McGeoch, 1990).

To compare with previously established alphaherpesvirus S segment homologies, FASTA comparisons between the seven groups of alphaherpesvirus-related sequences were conducted and are included in Table 2. In addition, the program GAP was used in similar pairwise comparisons to generate optimal alignments and an overall determination of the total percentage of identical and similar amino acids shared by any given pair of sequences (Table 2). As shown in Table 2, homology comparisons between MDV S segment ORFs and their alphaherpesvirus counterparts were comparable to those previously observed between established S component homologies.

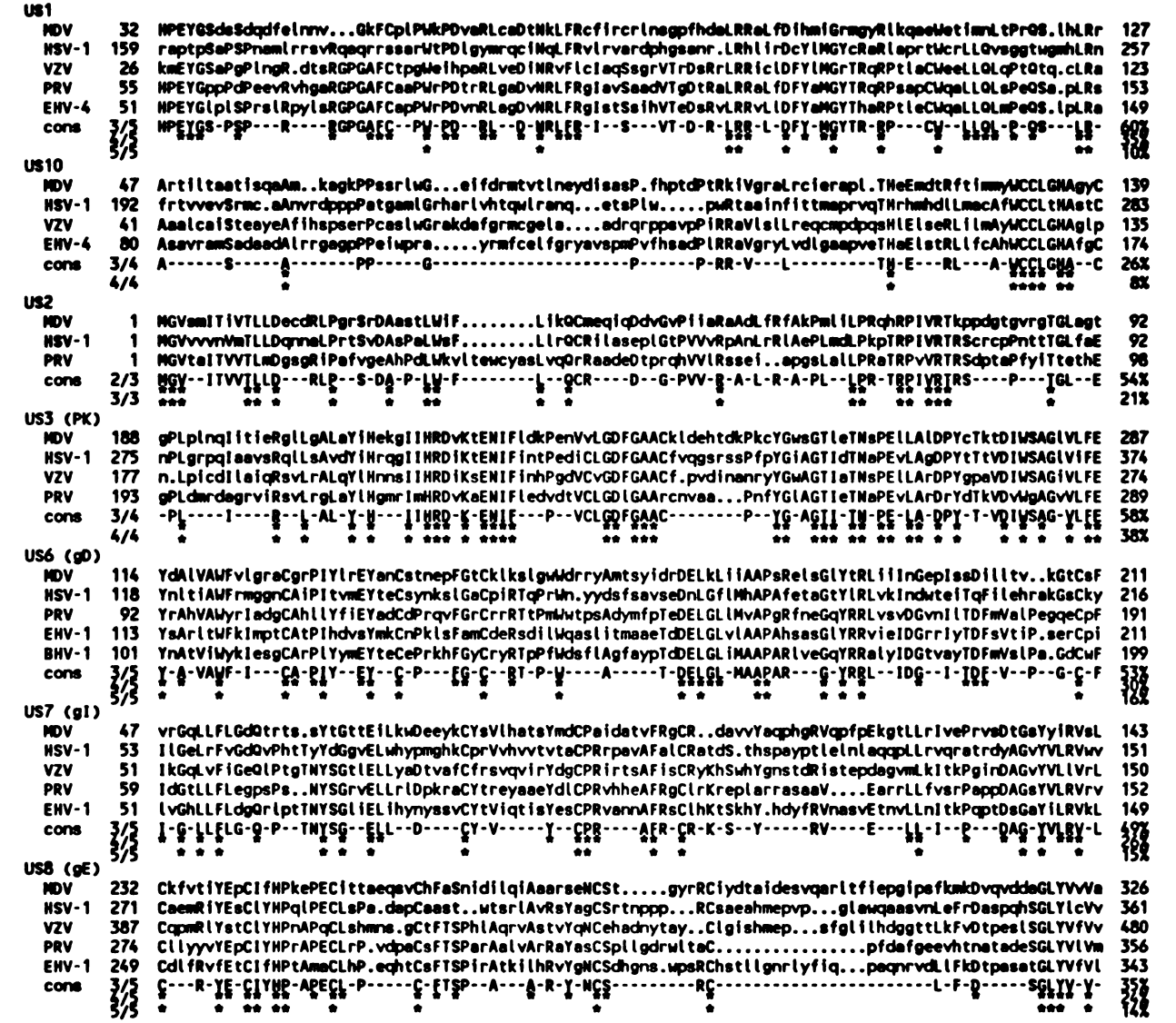
Figure 4A shows the results of limited multiple alignments for each of the seven alphaherpesvirus-homologous gene families that include an MDV counterpart. Amino acid regions (100 aa) showing greatest conservation are displayed and illustrate that common residues, particularly rare ones (e.g. cysteine, C and tryptophan, W), are often shared by all members of a given gene family. These amino acid conservation patterns are likely to contain functionally important domains with signiture motifs potentially useful for identification of distant viral and/or cellular counterparts as their sequences become available. Alternatively, these alignments point out potentially important

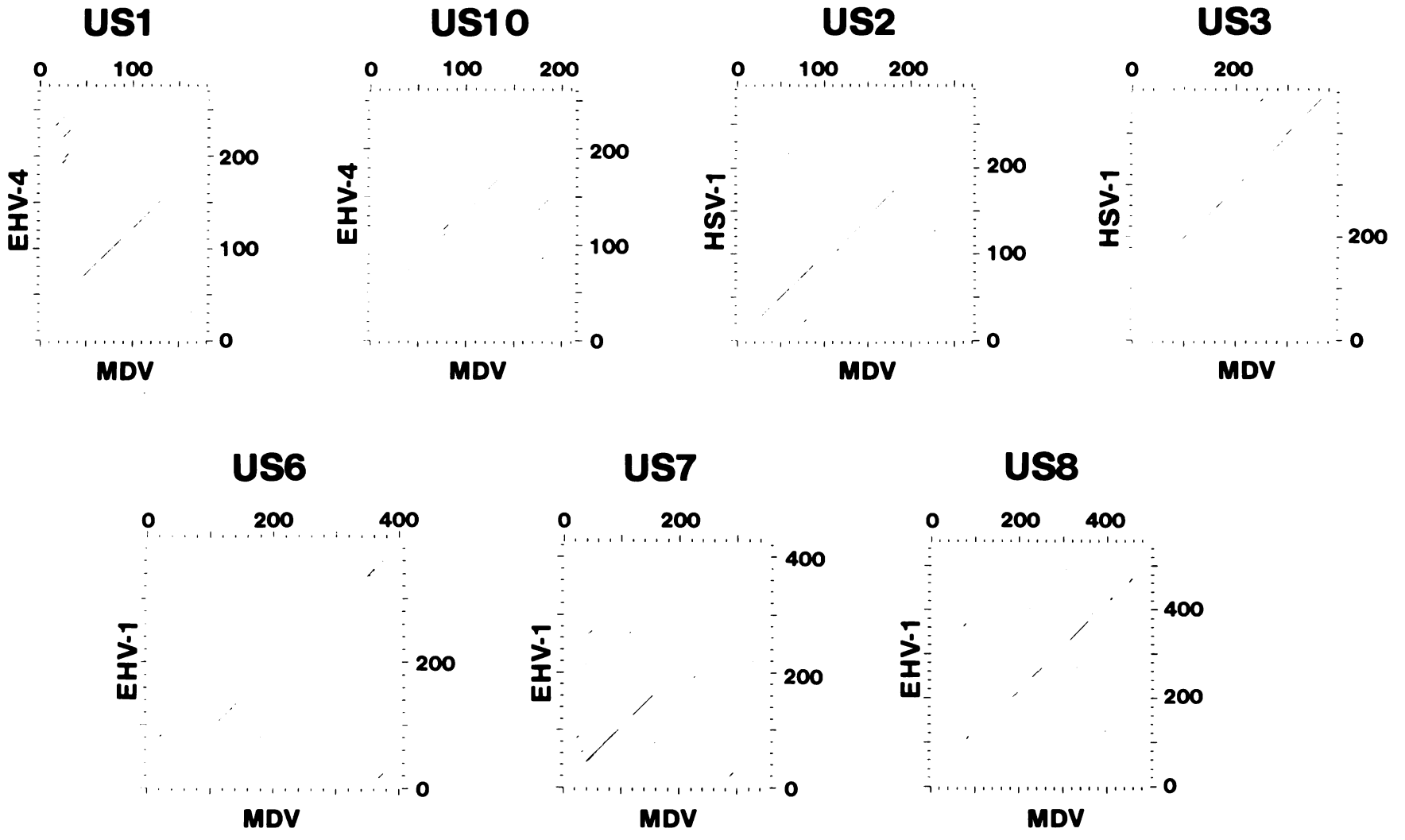
FIGURE 4. Comparison of alphaherpesvirus U_S homologies.

A) Alignment of S component homologs showing selected regions displaying maximum amino acid conservation using the UWGCG program, GAP. Some manual editing was performed to maximize alignment of identical and similar amino acids. The consensus sequence (cons) indicates residues that are shared by a given number of the virus' homologs as indicated; residues matching this number or greater are denoted with capital letters. Single- and double asterisks indicate residues conserved by 4/5, or all of the viruses, respectively. Amino acid numbers (with respect to 5'-ATG) of aligned sequences are listed at the beginning and end of each line.

B) Dot matrix homology plots generated using the UWGCG program DOTPLOT with the output file from UWGCG's COMPARE. The latter creates a file of the points of similarity between two predicted amino acid sequences for which a window length of 30 and a stringency of 15 were chosen (in which both identical and conservative amino acid replacements are scored positive). The resulting diagonal illustrates regions showing greatest conservation. Amino acid numbers (with respect to 5'-ATG) of corresponding sequences are denoted above and to the right of each plot.

FIGURE 4A




US1
0
100
200
EHV-4
MDV
US10
0
100
200
EHV-4
MDV
US2
0
100
200
HSV-1
MDV
US3
0
200
HSV-1
MDV
US6
0
200
400
EHV-1
MDV
US7
0
200
400
EHV-1
MDV
US8
0
200
400
EHV-1
MDV

amino acids to target for functional analysis employing site-directed mutagenesis.

Overall homologies were alternatively viewed using dot matrix homology plots (Figure 4B), in which conserved regions are depicted as diagonals. These conserved regions generally include, and in some cases extend upon, the regions depicted in Figure 4A. Taken together, these plots illustrate that the conservation of predicted amino acid sequences corresponding to U_S genes is often limited to particular regions, emphasizing their evolutionary divergence (Figure 4B).

Analysis of MDV glycoproteins, gD, gI and gE. In comparing the gB homologs of seven different herpesviruses included in the alpha-, beta- and gammaherpesvirus subfamilies, there is complete conservation of 10 cysteine residues (Ross et al., 1989). Alphaherpesvirus S component glycoproteins have also been found to contain similar patterns of conserved cysteine residues (McGeoch, 1990). HSV-1 US6 (gD) contains 7 cysteine residues, 6 appear critical for correct folding, antigenic structure and extent of oligosaccharide processing (Wilcox et al., 1988). Not only are these same 6 cysteines conserved among gD homologs of HSV-2 (McGeoch et al., 1987), PRV (Petrovskis et al., 1986a), EHV-1 (Audonnet et al., 1990; Flowers et al., 1991) and BHV-1 (Tikoo et al., 1990), but they are conserved by the MDV gD homolog as well (full alignment not shown). Similar cysteine conservation patterns apply to US7 (gI) and US8 (gE) homologs (McGeoch, 1990) Figure 4A depicts partial cysteine conservation patterns observed among the gD, gI and gE homologs, in which 4, 3 and 6 conserved cysteines are shown, respectively.

Careful inspection of the N-terminal regions of the MDV gD, gI and gE homologs has revealed that all contain the three basic building blocks of signal peptide sequences: a basic, positively charged N-terminal region (n-region), a

central hydrophobic region (h-region) and a more polar terminal region (c-region) that seems to define the cleavage site (von Heijne, 1985). Figure 2 shows the likely position of these sites using a recently improved method for predicting signal sequence cleavage sites (von Heijne, 1986). Also included are the location of other characteristic features of membrane glycoproteins, namely, the presence of potential N-glycosylation sites (i.e. N-X-S/T) and putative hydrophobic transmembrane and charged cytoplasmic domains near the C-terminal end. Like other gI homologs, MDV's counterpart contains a relatively long cytoplasmic domain. However, in contrast to the other gD homologs, MDV gD's signal peptide contains a longer n-region (18 residues), that is unusually highly charged (+4; Figure 2) considering an overall mean value of +1.7 among eukaryotes, which generally does not vary with length (von Heijne, 1986). Although a methionine codon exists directly before the hydrophobic h-region at position 6997 in Figure 2 (as PRV's gD homolog, Petrovskis et al., 1986a), the scanning model for translation (Kozak, 1989) favors usage of the more 5'-proximal initiation codon (at position 6943, Figure 2). Despite this prediction, a possible mRNA cap site location between these two ATG sites would preclude the more upstream initiation at position 6943.

Comparison of MDV sequences to those previously published. Comparison of sequences of the "virulent" GA strain of MDV (Figure 2) with those derived from a 5.5 kbp region of the "very virulent" RB1B strain of MDV (Ross et al., 1991) has revealed over 99% identity at both the nucleic acid and predicted amino acid levels. One difference results in an extension of five additional amino acids at the 5' end of the GA US6/gD (M-N-R-Y-R) relative to its RB1B sequence counterpart (ORF5; Ross et al., 1991). In addition to the 5 aa extension, the next four positions in the GA strain (Y-E-S-I) would differ from the corresponding RB1B positions (M-K-V-F). It would be interesting if these signal

sequences were found to differentially affect gD processing in these two strains. The GA US3 sequence is nearly identical to the RB1B US3 sequence, however the translational context criteria in this case would predict initiation most likely to begin with the 2nd in-frame methionine codon (position 10 of of the RBIB sequence). Our preliminary findings indicate that MDV US3 is expressed as two polypeptides (47/49 kDa), possibly consistent with initiation from both methionines (P. Brunovskis, unpublished observation). The predicted amino acid sequence of the GA US2 (Figure 2) is identical to that published in a recent report (Cantello et al., 1991), except for the presence of an alanine in place of an arginine at position 143. This minor difference is due to the inversion of a guanine and a cytosine relative to each other in the two GA sequences.

The RB1B counterpart of SORF4 (ORF4 in their report) was recently proposed to be a probable homolog of HSV-1 gG (Ross & Binns, 1991). It is tempting to propose such a homology, given their similar locations relative to other U_S region genes. We have further tested this proposed homology by similarly aligning these two sequences with GAP, following repeated shuffles of either of the two sequences while maintaining length and composition, (using the /RANdomizations command line option for 100 randomizations). This analysis was performed twice (each time with one of the two sequences shuffled). In doing so, we failed to find a significant difference in homology score ratios between the actual- versus randomized alignments (1.12 +/- 0.07). In some cases, the homologies of the randomized alignments actually exceeded the proposed MDV ORF4/HSV-1 gG alignment. Therefore we don't consider the proposed homology to be statistically significant. In fact, when using the type of stringency as in the above example, more significant homologies are observed following almost any database search involving a given ORF (data not shown). While we can not absolutely rule out that the two sequences are evolutionarily

related, any functional homology would appear absent, since the supposed MDV gG homolog lacks hydrophobic domains representing signal peptide- and transmembrane domain regions. Thus, it would appear that, at the very least, selection pressure for the maintainance of a common glycoprotein function appears to have been lost in this case.

DISCUSSION

In this report we have identified 3 new ORFs homologous to HSV US1 (ICP22), US8 (gE), and US10; 2 new MDV-specific U_S ORFs (SORF1 and -3), a fowlpox virus homolog (SORF2), and a complete HSV US7-homologous sequence. This extends upon the sequence analysis of a 5,255-bp segment located in the U_S region of the RB1B strain (Ross et al., 1991; Ross & Binns, 1991) and indicates only minor sequence differences in this region between two oncogenic serotype 1 strains, the "very virulent" RB1B- and the "virulent" GA strain. With completion of the entire 11,160-bp U_S sequence (GA strain), we have precisely determined the IR_S-U_S and U_S-TR_S junctions; these were somewhat of a surprise, since previous results using the same MDV strain as ours (GA) mapped the IR_S-TR_S junction to a different fragment located 2-3 kb upstream of this new location (Fukuchi et al., 1985). Completing the U_S sequence necessitated the characterization of entirely new MDV clones; these should now allow for the complete characterization of MDV's S region, or alternatively serve as probes for finding new homologs among avirulent serotype-2- (naturally avirulent MDV) or serotype-3 (HVT) strains.

Alphaherpesvirus S regions are characterized by a set of homologs which are specific to members of this taxonomic subfamily (Davison & Taylor, 1987; McGeoch, 1990). The identification of 7 alphaherpesvirus S region homologs in this study is consistent with MDV bearing a closer relation to alphaherpesviruses than gammaherpesviruses (Buckmaster et al., 1988). Furthermore, like other alphaherpesviruses, MDV's U_S region failed to show statistically significant CpG deficiencies that are common to all gammaherpesviruses thus far analyzed (Efstathiou et al., 1990; Honess et al., 1989).

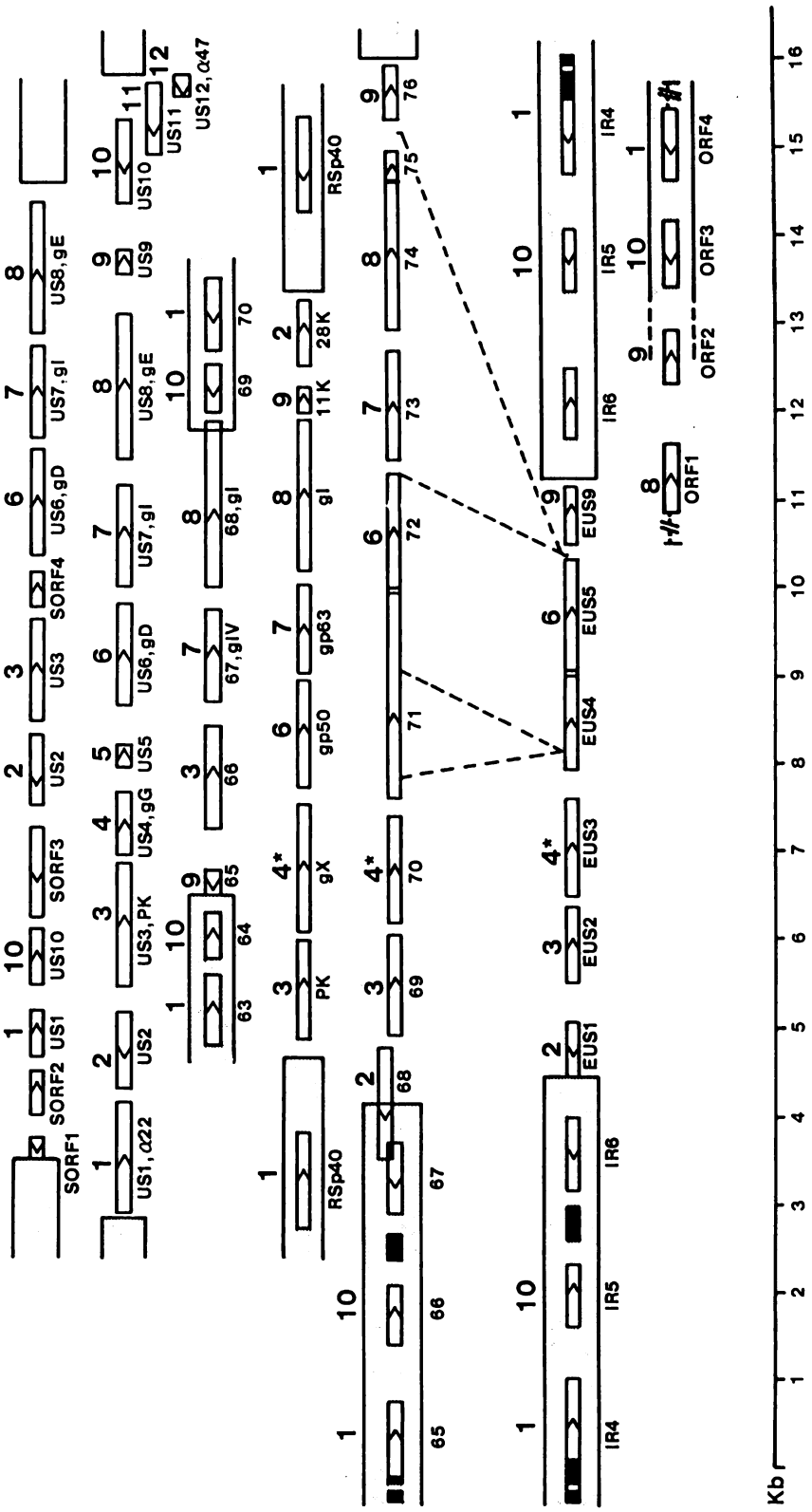
Molecular differences between MDV and alphaherpesvirus S components as a strategy for defining biological divergence and pathogenesis. Since MDV has been traditionally regarded as a gammaherpesvirus, much of the previous work interpreting MDV's properties has proceeded by analogy with the association between EBV and B cells (Wen et al., 1988, for example). Because of the closer genetic relationship between MDV and other alphaherpesviruses, we agree with others (Lawrence et al., 1990) that the lymphotropic properties of MDV and HVT are unlikely to be determined by molecules homologous to those of EBV. In fact, it can be argued that MDV has little in common with gammaherpesviruses, other than a capacity to latently infect and transform lymphocytes. Moreover, lymphotropism (and epitheliotropism) is probably common to all herpesviruses and is largely responsible for the widespread dissemination of HSV and VZV in cases involving neonatal and immunocompromised patients, often resulting in death (Nahmias & Roizman, 1973; Grose, 1982). These infections are characterized by a biphasic viremia similar to that observed in MDV-infected chickens; in the absence of maternal antibodies, young chickens can often die from an early mortality syndrome lacking any tumor involvement (Jakowski, et al., 1970; Witter, et al., 1980). Equine herpesvirus-1, an alphaherpesvirus, can also establish latent infections in T-lymphocytes (Welch et al., 1992). This lends support to an earlier proposal characterizing EHV-1 as a T-lymphotropic herpesvirus (Scott, et al., 1983). In addition to latent T-lymphocyte infections, MDV also appears to establish latent infections in both Schwann- and satellite cells (Pepose, et al., 1981) like VZV (Croen, et al., 1988). Such complexities suggest that a biologically-based classification system is overly simplistic, potentially misleading, and guided by biases dictated by the limited availability of systems to manipulate and study these viruses in vitro. Upon further examininination, more parallels exist between

the "lymphotropic" MDV and the "neurotropic" alphaherpesviruses than previously appreciated. To account for the different biological expressions that exist, a renewed focus on molecular differences between MDV and other alphaherpesviruses may be in order.

To account for such differences, the MDV U_S region (and adjoining repeats) may be particularly important. Fifty-three of the fifty-five unique long (U_L) region genes of HSV-1 possess an equivalent in VZV (McGeoch et al., 1988); a considerable number of these are related to beta- and gammaherpesvirus genes as well (29 of 67 EBV genes are counterparts to VZV U_L genes; Davison & Taylor, 1987). In contrast, alphaherpesvirus S components are specific for members of this taxonomic subfamily and appear to represent their most divergent coding region (Davison & Wilkie, 1983; Davison & McGeoch, 1986). In comparing MDV with other alphaherpesviruses, significant divergence also extends to the U_L-flanking repeat regions (Buckmaster et al., 1988) which are known to be expressed in tumor cells (Schat et al., 1989; Sugaya et al., 1991). A comparison of the genetic organization of alphaherpesvirus S segment genes is presented in Figure 5. Despite obvious similarities, there are marked differences in (i) gene content, organization, and localization; (ii) sequence conservation; and (iii) positioning of IR_S/U_S and U_S/TR_S junctions. Nevertheless, these overall gene layouts are consistent with a model to account for the divergence of alphaherpesviruses from a common ancestor by a number of homologous and semi-homologous recombination events which result in expansion or contraction of the inverted repeat regions and a concomitant loss or gain of U_S gene(s) (Davison & McGeoch, 1986). In the case of VZV, homologs of six HSV-1 U_S region genes are missing (US2, US4, US5, US6, US11, US12). Unlike all other alphaherpesviruses thus far analyzed (Figure 5), MDV appears to lack a US9 homolog. The HSV-1 US9 gene is

FIGURE 8. Comparison of MDV and alphaherpesvirus S component genes.

Based on published S region ORFs or unpublished ORFs accessible from GenBank (Audonnet et al., 1990; Breeden et al., 1992; Colle et al., 1992; Cullinane et al., 1988; Davison & Scott, 1986; Holden et al., 1992a, 1992b; McGeoch et al., 1985; Petrovskis et al., 1986a, 1986b; Petrovskis & Post, 1987; Telford et al., 1992; van Zijl et al., 1990; Zhang & Leader, 1990). Numbers above boxes refer to homologs based on relation to HSV-1 U_S ORF nomenclature (McGeoch et al., 1985). Polypeptide designations common to each system are listed below each of those boxes where applicable. Larger, stippled boxes refer to identified IR_S, TR_S, and/or R_S regions. Broken lines define regions deleted from the EHV-1 Kentucky A cell culture strain relative to that of the EHV-1 Ab4p field isolate strain. Bolded areas identify repeat sequences present in the two EHV-1 strains. Dashed lines extending from the stippled box of the EHV-4 TR_S region reflect uncertainty regarding the precise location of its U_S-TR_S junction (Cullinane et al., 1988). Asterisks refer to homologs which show relatedness to HSV-2 US4, rather than HSV-1 US4.


MDV
HSV-1
VZV
PRV
EHV-1 Ab4p
EHV-1 KyA
EHV-4
SORF1
SORF2
US1
US2
US3,PK
US4,gG
US5
US3
SORF4
US6,gD
US7,gI
US8,gE
US9
US10
US11
US12,α47
US1,α22
RSp40
PK
gX
gp50
gp63
67,gIV
68,gI
gI
11K
28K
74
75
76
73
72
71
70
69
68
67
66
65
64
63
EUS1
EUS2
EUS3
EUS4
EUS5
EUS9
IR4
IR5
IR6
ORF1
ORF2
ORF3
ORF4
Kb

known to encode a differentially phosphorylated 12-20 kDa tegument protein which becomes associated with nucleocapsids at or, soon after, their formation in the nuclei of infected cells (Frame et al., 1986). A recent study has suggested that PRV's US9 homolog has a function associated with envelopment at the nuclear membrane (Pol et al., 1991). Lacking such a homolog might explain MDV's characteristic inability to become stably enveloped in tissues other than the feather follicle epithelium.

Presence of MDV-specific and fowlpox virus-homologous genes. Unlike other alphaherpesviruses, MDV contains at least 3 MDV-specific ORFs in its U_S region SORFs 1, -3 and -4 (Figure 5). Virus-specific S component ORFs have also been identified in HSV-1 and -2 (US5, US11 and US12; Davison & McGeoch, 1986; McGeoch et al., 1985) and in EHV-1 (ORF67, 71, and 75, Telford et al., 1992; EUS4, Colle et al., 1992). Further sequence analysis of other alphaherpesvirus S regions will be necessary to confirm whether such genes are truly unique to given herpesviruses.

SORF3, located in the EcoRI-O subfragment (Figure 1B), specifies a 351 aa MDV-specific ORF. Considering its location, preliminary transcriptional mapping of the other genes mapping in EcoRI-O (e.g. MDV US1 and -10) (P. Brunovskis, unpublished observations) and previously reported data (Schat et al., 1989), it appears possible that SORF3 may code for the 1.1 kb A_j transcript, one of four immediate-early transcripts consistently identified in all MDV tumor cell lines tested (Schat et al., 1989).

A major surprise from this work was finding a FPV-related ORF. We are not aware of any other examples of such conservation across virus family lines, except a few cases that include cellular counterparts as well. MDV's FPV homolog, SORF2, was found to be 67% similar and 42% identical (over 100 aa) to FPV ORF4 (Tomley et al., 1988). With a Fast A score of 237 and the alignment

in Figure 3, the level of conservation is in many respects more striking than that which characterizes alphaherpesvirus S region homologies (Figure 4). Interestingly, compared with FPV ORF4, SORF2 contains an amino-terminal extension of 82 aa; conversely, ORF4 carries a carboxy-terminal extension of 41 aa. The block of conserved sequences may encode one or more functional domains that have independantly evolved following host cell-acquired gene transfer. On the other hand, it is intriguing to consider the possibility of virus-virus gene transfer. Individual cells have been found to be simultaneously cohabited by MDV and FPV (Tripathy et al., 1975). Given the different modes of replication for MDV and FPV (e.g. nuclear vs. cytoplasmic) such a possibility could point to a possibly novel form of gene transfer.

MDV U_S region genes as potential determinants for pathogenesis and tissue tropism. Recent studies have shown that 11 of 12 open reading frames contained in the HSV-1 U_S region are dispensable for growth *in vitro* (Longnecker et al., 1987; Roizman & Sears, 1990). These, and other "dispensable" genes appear to specify functions for optimal survival, maintenance and dissemination among the host (and its population at-large), rather than the presence of functions necessary for replication (Longnecker et al., 1987; Roizman & Sears, 1990). The significant divergence of alphaherpesvirus S components may reflect this region's capacity for determining distinct tissue- and host cell growth potentialities. Previous results have suggested that the product of HSV US1 (ICP22) encodes a determinant for tissue tropism, since its function appears to be dispensable for growth in some cell lines, but not others (Sears et al., 1985). Considering the extensive genetic divergence among a cluster of different glycoprotein homologs, each potentially subject to glycosylation, phosphorylation, palmitylation, myristylation and/or sulfation (Grose, 1990), a potentially large window exists for the creation of

multiply distinct virus-cell interactions which can affect host range, tissue tropism, invasiveness and cell-cell spread. Previous results have demonstrated that "nonessential" alphaherpesvirus glycoproteins encode functions associated with virulence (Lominiczi et al., 1984; Meignier et al., 1988; Mettenleiter et al., 1988; Roizman & Sears, 1990). This may reflect their ability to promote the infection and spread of virus in vivo (Lominiczi et al., 1984; Longnecker et al., 1987; Mettenleiter et al., 1988; Pol et al., 1991; Card et al., 1992). Consistent with this proposal is the observation that a specific deletion of PRV gI (homolog of HSV gE) was found to reduce the spread of infection in both rat (Card, et al., 1992) and pig (Kimman, et al., 1992) central nervous systems. This defect could reflect the inability of PRV gI mutants to promote cell-cell spread (Zsak et al., 1992).

Recent findings (M. Wild, personal communication) indicate that the HVT U_S region is no more than 7.5 kb and contains homologs of alphaherpesvirus U_S region genes; the latter result conflicts with an earlier hybridization-based report indicating a lack of homology between MDV and HVT U_S regions (Igarashi et al., 1987). A difference of at least 3.5 kb suggests that avirulence may reflect an absence of U_S homologs or other ORFs that are common to pathogenic MDV strains.

If MDV U_S region genes specify virulence determinants, these could indirectly affect oncogenic potential by affecting any number of critical events which precede tumor induction. Previous studies have shown that oncogenic potential appears to be directly correlated with cell-associated viremia levels and the capacity to cause immunosuppression (Calnek & Witter, 1991). The sequence of events leading to transformation include (i) an initial lytic growth phase in B cells, which is thought to cause activation and expansion of T-cells; (ii) latent growth phase involving infected T-cells; (iii) a second wave of lytic

infection, coincident with permanent immunosuppression; and (iv) oncogenic transformation (Calnek, 1986). Attenuated MDV strains (derived from oncogenic serotype 1 strains), as well as nononcogenic MDV and HVT stains (serotypes 2 and 3, respectively), are deficient in inducing the early cytolytic infection of B cells in chickens, suggesting that their cell tropisms differ from oncogenic strains (Schat et al., 1985; Shek et al., 1982). This is reflected in additional findings which show that attenuation of MDV leads to a marked reduction in infectivity and/or replication in lymphocytes (Schat et al., 1985).

In conclusion. The current herpesvirus classification system has been described as 'simple, fortuitously appropriate and defective' (Roizman, 1990b). It has been further suggested that 'the delineation and evolutionary relatedness of genes responsible for biological properties may be a more significant criterion for both evolutionary relatedness and classification than the arrangement and evolution of genes conserved throughout the family Herpesviridae, although they are not yet known' (Roizman, 1990b). While such a proposal is subject to debate, inasmuch as the alphaherpesvirus-specific U_S region is known to specify a cluster of supplementary essential functions thought to be important in defining biological properties, our sequence provides a foundation for further MDV studies aimed at identifying some of the genes responsible for the apparent discrepancy between MDV's genetic- and biologic properties.

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REFERENCES

Audonnet, J.-C., J. Winslow, G. Allen, and E. Paoletti. 1990. Equine herpesvirus type 1 unique short fragment encodes glycoproteins with homology to herpes simplex virus type 1 gD, gI and gE. J. Gen. Virol. **71**:2969-2978.

Buckmaster, A. E., S. D. Scott, M. J. Sanderson, M.E.G. Boursnell, N.L.J. Ross, and M. M. Binns. 1988. Gene sequence and mapping data from Marek's disease virus and herpesvirus of turkeys: implication for herpesvirus classification. J. Gen. Virol. **69**:2033-2042.

Calnek, B. W. 1986. Marek's disease, a model for herpesvirus oncology. Crit. Rev. Microbiol. **12**:293-320.

Calnek, B. W. and R. L. Witter. 1991. Marek's disease, p. 342-385. *In* B. W. Calnek (ed.), Diseases of poultry, Iowa State University Press.

Cantello, J. L., A. S. Anderson, A. Francesconi, and R. W. Morgan. 1991. Isolation of a Marek's disease virus (MDV) recombinant containing the lacZ gene of Escherichia coli stably inserted within the MDV US2 gene. J. Virol. **65**:1584-1588.

Card, J. P., M. E. Whealy, A. K. Robbins, and L. W. Enquist. 1992. Pseudorabies virus envelope glycoprotein gI influences both neurotropism and virulence during infection of the rat visual system. J. Virol. **66**:3032-3041.

Cebrian, J., C. Kaschka-Dierich, N. Berthelot, and P. Sheldrick. 1982. Inverted repeat nucleotide sequences in the genomes of Marek disease virus and the herpesvirus of the turkey. Proc. Natl. Acad. Sci. USA **79**:555-558.

Colle III, C., C. C. Flowers, and D. O'Callaghan. 1992. Open reading frames encoding a protein kinase, homolog of glycoprotein gX of pseudorabies virus, and a novel glycoprotein map within the unique short segment of equine herpesvirus type 1. Virology **188**:545-557.

Croen, K.D., J. M. Ostrove, L. J. Dragovic, and S. E. Straus. 1988. Patterns of gene expression are different for varicella-zoster and herpes simplex viruses. Proc. Natl. Acad. Sci. USA **85**:9773-9777.

Cullinane, A. A., F. J. Rixon, and A. J. Davison. 1988. Characterization of the genome of equine herpesvirus 1 subtype 2. J. Gen. Virol. **69**:1575-1590.

Davison, A. J. and N. M. Wilkie. 1983. Location and orientation of homologous sequences in the genomes of five herpesviruses. J. Gen. Virol. **64**:1927-1942.

Davison, A. J. and D. J. McGeoch. 1986. Evolutionary comparisons of the S segments in the genomes of herpes simplex virus type 1 and varicella-zoster virus. J. Gen. Virol. **67**:597-611.

Davison, A. J. and J. E. Scott. 1986. The complete DNA sequence of varicella-zoster virus. J. Gen. Virol. **67**:1759-1816.

Davison, A. J. and P. Taylor. 1987. Genetic relations between varicella-zoster virus and Epstein-Barr virus. J. Gen. Virol. **68**:1067-1079.

Devereaux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. Nucl. Acids. Res. **12**:387-395.

Efstathiou, S., Y. M. Ho, S. Hall, C. J. Styles, S. D. Scott, and U. A. Gompels. 1990. Murine herpesvirus 68 is genetically related to the gammaherpesviruses Epstein-Barr virus and herpesvirus saimiri. J. Gen. Virol. **71**:1365-1372.

Flowers, C. C., E. M. Eastman, and D. J. O'Callaghan. 1991. Sequence analysis of a glycoprotein D gene homolog within the unique short segment of the EHV-1 genome. Virology. **180**:175-184.

Frame, M. C., D. J. McGeoch, F. J. Rixon, A. C. Orr, and H. S. Marsden. 1986. The 10K virion phosphoprotein encoded by gene US9 from herpes simplex virus type 1. Virology. 150:321-332.

Fukuchi, K., M. Sudo, Y.-S. Lee, A. Tanaka, and M. Nonoyama. 1985. Structure of Marek's disease virus DNA: detailed restriction enzyme map. J. Virol. 51:102-109.

Gibbs, C. P., K. Nazerian, L. F. Velicer, and H.-J. Kung. 1984. Extensive homology exists between Marek disease herpesvirus and its vaccine virus, herpesvirus of turkeys. Proc. Natl. Acad. Sci. USA 81:3365-3369.

Grose, C. 1982. Varicella-zoster infections: chickenpox (varicella) and shingles (zoster), p. 85-150. *In* Glaser, R. and T. Gotlieb-Stematsky (eds.), Human herpesvirus infections. Marcel Dekker Inc., New York.

Grose C. 1990. Glycoproteins encoded by varicella-zoster virus: biosynthesis, phosphorylation, and intracellular trafficking. Annu. Rev. Microbiol. 44:59-80.

Hanks, S. K., A. M. Quinn, and T. Hunter. 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science 241:42-52.

Holden, V. R., R. R. Yalamanchili, R. N. Harty, and D. J. O'Callaghan. 1992a. ICP22 homolog of equine herpesvirus 1: expression from early and late promoters. J. Virol. 66:664-673.

Holden, V. R., R. R. Yalamanchili, R. N. Harty, and D. J. O'Callaghan. 1992b. Identification and characterization of an equine herpesvirus 1 late gene encoding a potential zinc finger. Virology 188:704-713.

Honess, R. W. 1984. Herpes simplex and 'the herpes complex': diverse observations and a unifying hypothesis. J. Gen. Virol. 65:2077-2107.

Igarashi, T., M. Takahashi, J. Donovan, J. Jessip, M. Smith, K. Hirai, A. Tanaka, and M. Nonoyama. 1987. Restriction enzyme map of herpesvirus of turkey DNA and its collinear relationship with Marek's disease virus DNA. Virology. 157:351-358.

Jakowski, R. M., T. N. Fredrickson, T. W. Chomiak, and R. E. Luginbul. 1970. Hematopoietic destruction in Marek's disease. Avian Dis. 14:374-385.

Kimman, T. G., N. de Wind, N. Oei-Lie, J.M.A. Pol, A. J. Berns, and A.L.J. Gielkens. 1992. Contribution of single genes within the unique short region of Aujeszky's disease virus (suid herpesvirus type 1) to virulence, pathogenesis and immunogenicity. J. Gen Virol. 73:243-251.

Kozak, M. 1989. The scanning model for translation: an update. J. Cell Biol. 108:229-241.

Lawrence, G. L., M. Chee, M. A. Craxton, U. A. Gompels, R. W. Honess, and B. G. Barrell. 1990. Human herpesvirus 6 is closely related to human cytomegalovirus. J. Virol. 64:287-299.

Lomniczi, B., S. Watanabe, T. Ben-Porat, and A. S. Kaplan. 1984. Genetic basis of the neurovirulence of pseudorabies virus. J. Virol. 52:198-205.

Longnecker, R., S. Chatterjee, R. J. Whitley, and B. Roizman. 1987. Identification of a herpes simplex virus 1 glycoprotein gene within a gene cluster dispensable for growth in cell culture. Proc. Natl. Acad. Sci. USA 84:4303-4307.

McGeoch, D. J., A. Dolan, S. Donald, and F. J. Rixon. 1985. Sequence determination and genetic content of the short unique region in the genome of herpes simplex virus type 1. J. Mol. Biol. 181:1-13.

McGeoch, D. J., H.W.M. Moss, D. McNab, and M. C. Frame. 1987. DNA sequence and genetic content of the HindIII L region in the short unique component of the herpes simplex virus type 2 genome: identification of

the gene encoding glycoprotein G, and evolutionary comparisons. J. Gen. Virol. 68:19-38.

McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor. 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. 69:1531-1574.

McGeoch, D. J. 1990. Evolutionary relationships of virion glycoprotein genes in the S region of alphaherpesvirus genomes. J. Gen. Virol. 71:2361-2367.

Meignier, B., R. Longnecker, P. Mavromara-Nazos, A. E. Sears, and B. Roizman. 1988. Virulence of and establishment of latency by genetically engineered deletion mutants of herpes simplex virus 1. Virology 162:251-254.

Mettenleiter, T. C., C. Schreurs, F. Zuckerman, T. Ben-Porat, and A. S. Kaplan. 1988. Role of glycoprotein gIII of pseudorabies virus in virulence. J. Virol. 62:2712-2717.

Nahmias, A. J. and B. Roizman. 1973. Infection with herpes-simplex virus 1 and 2. N. Engl. J. Med. 289:781-789.

Pearson, W. R. and D. J. Lipman. 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA 85:2444-2448.

Pepose, J. S., J. G. Stevens, M. L. Cook, and P. W. Lampert. 1981. Marek's disease as a model for the Landry-Guillain-Barre Syndrome: latent viral infection in nonneuronal cells is accompanied by specific immune responses to peripheral nerve and myelin. Am. J. Pathol. 103:309-320.

Petrovskis, E. A., J. G. Timmins, M. A. Armentrout, C. C. Marchioli, R. J. Yancey, Jr., and L. E. Post. 1986a. DNA sequence of the gene for pseudorabies virus gp50, a glycoprotein without N-linked glycosylation. J. Virol. 59:216-223.

Petrovskis, E. A., J. G. Timmins, and L. E. Post. 1986b. Use of lambda gt11 to isolate genes for two pseudorabies virus glycoproteins with homology to herpes simplex virus and varicella-zoster virus glycoproteins. J. Virol. 60:185-193.

Petrovskis, E. A., and L. E. Post. 1987. A small open reading frame in pseudorabies virus and implications for evolutionary relationships between herpesviruses. Virology 159:193-195.

Pol, J.M.A., F. Wagenaar, and A. Gielkens. 1991. Morphogenesis of three pseudorabies virus strains in porcine nasal mucosa. Intervirology. 32:327-337.

Rauh, I. and T. C. Mettenleiter. 1991. Pseudorabies virus glycoproteins gII and gp50 are essential for virus penetration. J. Virol. 65:5348-5356.

Rixon, F. J. and D. J. McGeoch. 1985. Detailed analysis of the mRNAs mapping in the short unique region of herpes simplex virus type 1. Nucl. Acids Res. 13:953-973.

Roizman, B., L. E. Carmichael, F. Deinhardt, G. de-The, A. J. Nahmias, W. Plowright, F. Rapp, P. Sheldrick, M. Takahashi, and K. Wolf. 1981. Herpesviridae: definition, provisional nomenclature, and taxonomy. Intervirology. 16:201-217.

Roizman, B. 1990a. Whither herpesviruses?, p 285-291. *In* C. Lopez et al. (ed.), Immunobiology and prophylaxis of human herpesvirus infections. Plenum Press, New York.

Roizman, B. 1990b. Herpesviridae: a brief introduction, p. 1787-1793. *In* B. N. Fields et al. (ed.), Virology, 2nd ed., vol. 2. Raven Press, New York.

Roizman, B and A. E. Sears. 1990. Herpes simplex viruses and their replication, p. 1795-1841. *In* B. N. Fields et al. (ed.), Virology, 2nd ed., vol. 2. Raven Press, New York.

Ross, L.J.N., M. Sanderson, S. D. Scott, M. M. Binns, T. Doel, and B. Milne. 1989. Nucleotide sequence and characterization of the Marek's disease virus homologue of glycoprotein B of herpes simplex virus. J. Gen. Virol. **70**:1789-1804.

Ross, L.J.N., and M. M. Binns. 1991. Properties and evolutionary relationships of the Marek's disease virus homologues of protein kinase, glycoprotein D and glycoprotein I of herpes simplex virus. J. Gen. Virol. **72**:939-947.

Ross, L.J.N., M. M. Binns, and J. Pastorek. 1991. DNA sequence and organization of genes in a 6.6 kbp Eco RI fragment mapping in the unique short segment of Marek's disease virus (strain RB1B). J Gen. Virol. **72**:949-954.

Sanger, F. S., S. Nicklin, and A. R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463-5467.

Schat, K. A., B. W. Calnek, J. Fabricant, and D. L. Graham. 1985. Pathogenesis of infection with attenuated Marek's disease virus strains. Avian Pathol. **14**:127-146.

Schat, K. A., A. Buckmaster, and L.J.N. Ross. 1989. Partial transcriptional map of Marek's disease herpesvirus in lytically infected cells and lymphoblastoid cell lines. Int. J. Cancer. **44**:101-109.

Scott, S. D., N.L.J. Ross, and M. M. Binns. 1989. Nucleotide and predicted amino acid sequences of the Marek's disease virus and turkey herpesvirus thymidine kinase genes; comparison with thymidine kinase genes of other herpesviruses. J. Gen. Virol. **70**:3055-3065.

Shek, W. R., K. A. Schat, and B. W. Calnek. 1982. Characterization of non-oncogenic Marek's disease virus-infected and turkey herpesvirus-infected lymphocytes. J. Gen. Virol. **63**:333-241.

Sugaya, K., G. Bradley, M. Nonoyama, and A. Tanaka. Latent transcripts of Marek's disease virus are clustered in the short and long repeat regions. J. Virol. **64**:5773-5782.

Telford, E.A.R., M. S. Watson, K. McBride, and A. J. Davison. 1992. The DNA sequence of equine herpesvirus-1. Virology 189:304-316.

Tikoo, S. K., D. R. Fitzpatrick, L. A. Babiuk, and T. J. Zamb. 1990. Molecular cloning, sequencing, and expression of functional bovine herpesvirus 1 glycoprotein gIV in transfected bovine cells. J. Virol. **64**:5132-5142.

Tomley, F., M. Binns, J. Campbell, and M. Boursnell. 1988. Sequence analysis of an 11.2 kilobase, near-terminal, BamHI fragment of fowlpox virus. J. Gen. Virol. **69**:1025-1040.

Tripathy, D. N., D. M. Sells, and L. E. Hanson. 1975. Natural pox and herpes as a dual viral infection in chickens. Avian Dis. **19**:75-81.

van Zijl, M., H. van der Gulden, N. de Wind, A. Gielkens, and A. Berns. 1990. Identification of two genes in the unique short region of pseudorabies virus; comparison with herpes simplex virus and varicella-zoster virus. J. Gen Virol. **71**:1747-1755.

von Heijne, G. 1985. Signal sequences. The limits of variation. J. Mol. Biol. **184**:99-105.

von Heijne, G. 1986. A new method for predicting signal sequence cleavage sites. Nucl. Acids Res. **14**:4683-4690.

Welch, H. M., C. G. Bridges, A. M. Lyon, L. Griffiths, and N. Edington. 1992. Latent equid herpesviruses 1 and 4: detection and distinction using the polymerase chain reaction and cocultivation from lymphoid cells. J. Gen. Virol. **73**:261-268.

Wilcox, W. C., D. Long, D. L. Sodora, R. J. Eisenberg, and G. H. Cohen. 1988. The contribution of cysteine residues to antigenicity and extent of

processing of herpes simplex virus type 1 glycoprotein D. J. Virol. 62:1941-1947.

Witter, R. L., J. M. Sharma, and A. M. Fadly. 1980. Pathogenicity of variant Marek's disease virus isolants in vaccinated and unvaccinated chickens. Avian Dis. 24:210-232.

Zhang, G. and D. P. Leader. 1990. The structure of the pseudorabies virus genome at the end of the inverted repeat sequences proximal to the junction with the short unique region. J. Gen. Virol. 71:2433-2441.

Zsak, L., F. Zuckermann, N. Sugg, and T. Ben-Porat. 1992. Glycoprotein gI of pseudorabies virus promotes cell fusion and virus spread via direct cell-to-cell transmission. J. Virol. 66:2316-2325.

Chapter III

Marek's disease virus expresses an unusual 27-kDa late class cytoplasmic phosphoprotein homologous to the 68-kDa herpes simplex 1 immediate-early nuclear phosphoprotein, ICP22

ABSTRACT

Marek's disease virus (MDV) is a highly cell-associated avian herpesvirus that can cause T cell lymphomas as quickly as three weeks postinfection (for a recent review, see Calnek and Witter, 1991). The recent knowledge that Marek's disease virus is phylogenetically more related to alphaherpesviruses than gammaherpesviruses raises interesting questions regarding the seeming incongruence between herpesvirus genetic- and biologic properties. To examine the nature of these differences, we have begun to focus our attention on the MDV S region, inasmuch as alphaherpesvirus S regions are known to contain clusters of genes specifying functions associated with pathogenesis and viral dissemination instead of those essential for virus production. In this study we have characterized the MDV homolog of HSV-1 ICP22, an immediate-early polypeptide thought to specify a determinant for tissue tropism. Northern blot analysis of RNA from MDV-infected cells identified a 1.9 kb PAA-sensitive US1 transcript. S1-nuclease protection analysis mapped its 5' and 3' ends to sites located near TATA- and poly $(A)^+$ consensus sequences; the length of the S1-protected region was consistent with that of the full-length transcript. Protein studies were carried out by immunoprecipitation analysis of PMSF-treated lysates from MDV-infected cells using monospecific, polyclonal antibodies generated from rabbits immunized with bacterially-synthesized *trpE*-US1 fusion proteins. In the absence of multiple protease inhibitors, the MDV US1 polypeptide was characterized as a 27-kDa MDV US1-encoded phosphoprotein, designated pp27; in their presence, a less abundant 24-kDa phosphoprotein was coprecipitated. In contrast to the 68-kDa nuclear HSV ICP22, MDV pp27 synthesis was almost completely dependent on viral DNA replication and its localization was restricted to the cytoplasm of infected DEF cell cultures. Pulse-chase experiments identified at least three distinct polypeptides of

approximately 27-kDa that were characterized by reduced mobility with longer chase periods. A larger, diffuse band approximately double in size (53-kDa) was observed to undergo a similar processing pattern. The 53-kDa polypeptides appeared to bear a direct relation to pp27,24 since its polypeptides could be precipitated from lysates boiled in the presence of 2-mercaptoethanol and SDS under conditions that led to the loss of all non-specifically precipitated polypeptides. Identification of the larger phosphoprotein(s) was enhanced following electrophoresis under non-reducing conditions; together these findings suggest that pp27,24 forms disulfide-linked dimers under native conditions.

INTRODUCTION

Marek's disease virus (MDV) is an acutely transforming herpesvirus which causes T-cell lymphomas in chickens. Marek's disease (MD) isolates have also been shown to cause lymphoproliferative neural lesions, peripheral nerve demyelination, blindness. and paralysis (for review, see Calnek and Witter, 1991). Based on its lymphotropic properties, Marek's disease virus (MDV) has been classified as a gammaherpesvirus (Roizman et al., 1981). However, recent evidence (Brunovskis and Velicer, 1992a; Buckmaster et al., 1988; Ross et al., 1989; Ross and Binns, 1991; Ross et al., 1991; Scott et al., 1989) indicates that MDV bears a much closer phylogenetic relationship to alphaherpesviruses, characterized by its prototype, herpes simplex virus (HSV).

MDV is known to contain at least 7 alphaherpesvirus-related genes in its unique short (U_S) region (Brunovskis and Velicer, 1992a). Alphaherpesvirus U_S (and other S region) genes are unique to members of this taxonomic subfamily (Davison and McGeoch, 1986; Davison and Taylor, 1987; McGeoch, 1990). They are particularly noted for encoding a cluster of genes that are nonessential for growth in cell culture (de Wind et al., 1990; Longnecker and Roizman, 1987; Longnecker et al., 1987; Sears et al., 1985; Weber et al., 1987). Unlike their wild-type parents, HSV U_S region gene mutants grow poorly in animal host models and are consistently associated with reduced levels of virulence and/or the capacity to induce latency (Meignier et al., 1988). The strikingly different biologic- and pathologic expressions that characterize herpesviruses are largely thought to be determined by the nonessential or *supplementary essential* genes which confer functions enabling efficient dissemination and growth in various tissues in the face of an immune system poised for its elimination (for discussion, see Roizman, 1990).

Unlike the L region, which appears to exhibit significantly greater conservation (McGeoch et al., 1988) and specifies genes shared by representatives of all three herpesvirus subfamilies (Davison and Taylor, 1987; Kouzarides et al., 1987), alphaherpesvirus S regions are substantially more diverse (Brunovskis and Velicer, 1992a; Davison and Taylor, 1987). This is apparent from both genetic organization- and sequence homology levels. HSV-1 ICP22 and its related alphaherpesvirus counterparts exhibit significant differences in length, amino acid (aa) conservation, and transcriptional kinetics (Holden et al., 1992; Zhang and Leader, 1990; M. Schwyzer, pers. comm.). Unlike other HSV immediate-early (IE) proteins, such as ICP4, the role of ICP22 is comparatively obscure. Evidence suggests that the ICP22-encoding gene of HSV-1 (i.e. US1) specifies a tissue tropism determinant (Sears et al., 1985). Although the 68-kDa ICP22 polypeptide is dispensable for growth in some cell lines, ICP22 mutants grow very poorly in others. This has led to the suggestion that certain cell lines (e.g. Vero) contain a host ICP22-like function that allows for the mutant to grow (Sears et al., 1985). Such a function may be related to gene regulation. Defects in ICP22 expression are associated with a reduction in late (L) gene expression (Epstein and Jacquemont, 1983; Jacquemont et al., 1984; Sears et al., 1985). VZV's ICP22 homolog was recently reported to repress the IE promoter of its ICP4 homolog, but stimulate the early (E) promoter of its TK homolog (Jackers et al., 1992).

Specific immunological reagents for ICP22-related proteins have only been created for HSV-1; however, while this synthetic peptide-derived antiserum was able to react with full-length and truncated ICP22 derivatives by Western blot analysis, the antiserum was unable to immunoprecipitate these polypeptides (Ackermann et al., 1985). In this study we have characterized MDV US1 expression at the transcriptional level by Northern blot- and S1 nuclease

protection analyses and at the translational level by immunoprecipitation studies using polyclonal antisera specific for the polypeptide encoded by MDV US1. Compared with its HSV-1 ICP22 counterpart, the MDV homolog exhibited a number of striking contrasts of potential significance to MDV's unique biological properties.

MATERIALS AND METHODS

Cells and viruses. The preparation of primary duck embryo fibroblast (DEF) cells was by established methods (Solomon, 1975). Cells were seeded in 100-mm diameter plastic tissue culture plates or large roller bottles (for RNA isolations) with $1x10^7$ or $4x10^8$ cells, respectively and a Medium 199/nutrient mixture F-10 combination containing 4% calf serum (CS). Pathogenic MDV GA strain-infected cells (passage level 6) and HVT vaccine virus strain FC-126-infected cells (passage level 13) frozen in liquid nitrogen were used to infect 85-90% confluent DEF cell monolayers at a 1:6-1:8 dilution in growth medium containing 4% CS. The following day and thereafter, virus-infected cells were maintained in growth medium containing 0.2-1.0% CS. In some experiments, cells were treated with cycloheximide (CHX; 100 μg/ml) or phosphonoacetic acid (PAA; 200 μg/ml) beginning 2 hr before- or 24 hr after infection, respectively.

Antisera. Immune chicken sera (ICS) was pooled together from convalescent chickens naturally exposed to MDV. 2BN90.1, a nuclear antigen-specific monoclonal antibody, was kindly provided by L. Lee (USDA Avian Disease and Oncology Laboratory, E. Lansing, MI).

RNA isolation and Northern blot analysis. Total cellular- and poly $(A)^+$-purified RNA (via oligo (dT)-cellulose chromatography) was isolated from mock- and virus-infected DEF cells by the guanidinium isothiocyanate procedure as previously described (Sithole et al., 1988), fractionated on formaldehyde-1.2% agarose gels and blotted onto nitrocellulose or nylon-based membranes by standard methods (Maniatis et al., 1982). More recent RNA isolations were carried out by the acid-guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). Probes were labeled with ^{32}P-dCTP, initially by nick-translation

and more recently by the random priming method, according to instructions supplied by the manufacturer. Standard hybridization and wash conditions were employed (Maniatis et al., 1982).

S1-nuclease protection analysis. S1-nuclease protection analysis was performed as described elsewhere (Maniatis et al., 1982) using 1 μg of poly $(A)^+$ mRNA per reaction. Probes were prepared by digestion of pBSM-O with AvaI followed by 5'- or 3' (end)labeling with polynucleotide kinase or Klenow fragment, respectively. The 5'- and 3'-labeled probes were gel-purified from 0.7% low melting point agarose gels following secondary digestions with EcoRI or ClaI, respectively. A 3' end-labeled (Klenow) pBR322/HinfI digest was used as a molecular weight standard.

Expression and purification of fusion protein immunogens. The 1.8 kb NcoI-ClaI fragment of pBSM-O was blunt-ended at the NcoI site with Klenow fragment and subcloned into SmaI/ClaI-digested expression vector, pATH2 (kindly provided by R. C. Schwartz) using $CaCl_2$-competent *E. coli* RR1 cells. The resulting construct allows for the NH_2-terminal 323-aa of *trpE* fused (by way of an inserted multiple cloning site) with MDV US1 (aa 56-172; Brunovskis and Velicer, 1992a). Induced expression and purification of *trpE*-US1 was according to a procedure recently described (Koerner et al., 1991).

Immunizations with *trpE*-US1 fusion proteins. 1.0-1.5 mg of 1x sample buffer-solubilized *trpE*-US1 was electrophoresed through a 3-mm thick 7.5% SDS-PAGE gel (preparative) and stained with Coomasie Blue. The entire strip containing *trpE*-US1 was excised, washed in distilled water for 1 hr and homogenized in 1-2 volumes of Freund's complete- or incomplete adjuvant by serial passage through 18-, 20-, 22- and 25-gauge syringe needles. Primary injections (subcutaneous route) of female New Zealand white rabbits involved the use of 750 μg of fusion protein in Freund's complete adjuvant; subsequent

injections employed 375 μg of fusion protein in Freund's incomplete adjuvant. The rabbits were boosted once a month; serum was collected 10-14 days following each boost.

Radiolabeling of proteins. Mock- and virus-infected DEF cells were labeled with 50 μCi/ml of [^{35}S]-methionine (specific activity, 1,000 μCi/mmol, ICN) at 48-72 hrs. post-infection for 2-4 hrs. as previously described (Isfort et al., 1986) with the following modifications. In later experiments, lysis buffers containing phenylmethylsulfonyl fluoride (PMSF) were supplemented with the following additional protease inhibitors; 50 μg/ml tosyl-lysyl-chloromethyl ketone (TLCK), 50 μg/ml tosylamide-phenylmethyl-chloromethyl ketone (TPCK), and 2 μg/ml aprotinin. DEF cells were similarly labeled with ^{32}P-orthophosphate with the following modifications. Phosphate-free Dulbecco modified Eagle medium was used for 3 hr before- and during the 2 hr labeling period in which 250 μCi/ml $^{32}P_i$ (carrier-free; Amersham Corp.) was used per 60 mm plate. Pulse-chase labeling with [^{35}S]-methionine was done as previously described (Chen et al., 1992), in this case employing a 5 min pulse followed by chases of 0, 5, 15, 30, 60, 180 and 300 min.

Immunoprecipitation and SDS-PAGE analyses. Immunoprecipitation/SDS-PAGE analyses were carried out as previosly described (Isfort et al., 1986). The specificity of *trpE*-US1-derived antisera was tested by preincubating 970 μl PBS with 20 μl 1x sample buffer-solubilized *trpE*-US1 and 10 μl US1-specific antisera (or preimmune control serum) for 30 min at 4 C. Labeled cell lysates were then added and processed as before. In some cases, lysates were adjusted to 1% SDS and 1% 2-mercaptoethanol (2-ME) and boiled for 5 min prior to the addition of antibodies (Simek and Rice, 1988). Immunoprecipitates were washed 2x with 1x phospholysis buffer (PLB) and electrophoresed on 10% SDS-PAGE gels. ^{3}H-

labeled protein markers (BRL) were included to allow for molecular weight estimations.

Subcellular fractionation. Cells from 100-mm mock- and GA-infected plates were labeled with [^{35}S]-methionine when extensive cytopathic effects were observed (72 hr p.i.), washed 2x with ice-cold PBS (pH 7.2), and scraped from plates with 1 ml ice-cold PBS (pH 7.2). Cells were precipitated in a microfuge (1000g, 10 min) and treated for 10 min on ice with 1 ml NP40 lysis buffer containing PMSF, TPCK, TLCK, and aprotinin. Nuclei were pelleted 10 min at 1,000g, and the cytoplamic supernatant fraction was retained. The nuclei were again washed with 1 ml NP40 lysis buffer, and pelleted as before. Nuclei were disrupted by the addition 1 ml of 1x PLB containing the above protease inhibitors. Whole cell fractions were obtained by substituting the NP40 lysis buffer addition step (above) with 1 ml 1x PLB as above. The three fractions were adjusted to equal volumes using 1x PLB. 50 μl of lysate were used for each immune-precipitation.

RESULTS

Northern blot- and S1-nuclease protection analyses of MDV US1. Prior to the identification of US1-related coding sequences by nucleotide sequencing, transcriptional mapping studies employing a radiolabeled EcoRI-O probe (Figure 1B; Silva and Witter, 1985) identified a 1.9 kb rightwardly-directed transcript (Figure 2A, lane 2; Figure 2B, lane 4). In DEF cells treated with PAA, an inhibitor of MDV DNA replication (Lee et al., 1976), the 1.9 kb transcript was reduced to scarcely detectable levels (Figure 2C, lanes 6,7). Identical results were subsequently obtained using a probe limited to MDV US1 coding sequences (data not shown). Expression of an MDV late control gene, gp57-65 (Coussens and Velicer, 1988) was found to be similarly affected by the addition of PAA (Figure 2, lanes 9,10). The reduced RNA levels were specifically attributable to PAA inhibition, rather than differences in the level of RNA loaded, inasmuch as similar levels of 18S and 28S rRNA were observed in the PAA+ and PAA- lanes following ethidium bromide staining of the gel prior to Northern transfer (data not shown). With longer exposures, the 1.9 kb US1 transcript could be detected at a low level in PAA-treated cells as well, suggesting a gamma-1 (leaky-late) mode of temporal regulation. However, in the absence of true one-step growth conditions for growth of MDV in cell culture, it is not clear whether the "PAA-insensitive" transcripts may have been true late (gamma-2) transcripts present in the infected cell inoculum used to infect new cells prior to the addition of PAA.

To map the precise location of the 1.9 kb transcript, an S1 nuclease-protection experiment was performed. Following hybridization of the 5'-labeled 0.72 kb EcoRI-AvaI probe (Probe I, Figure 3C) to 1 μg of MDV-infected poly $(A)^+$ and subsequent digestion with S1 nuclease, two fragments of 510 and 522

FIGURE 1. Localization of the 1.9 kb MDV Eco RI-O transcript.

A) MDV genome structure and BamHI restriction map.

B) Eco RI and Bam HI restriction maps identifying the area of focus.

C) Localization and polarity of the 1.9 kb Eco RI-O transcript.

LOCALIZATION OF THE 1.9 kb EcoRI-O TRANSCRIPT

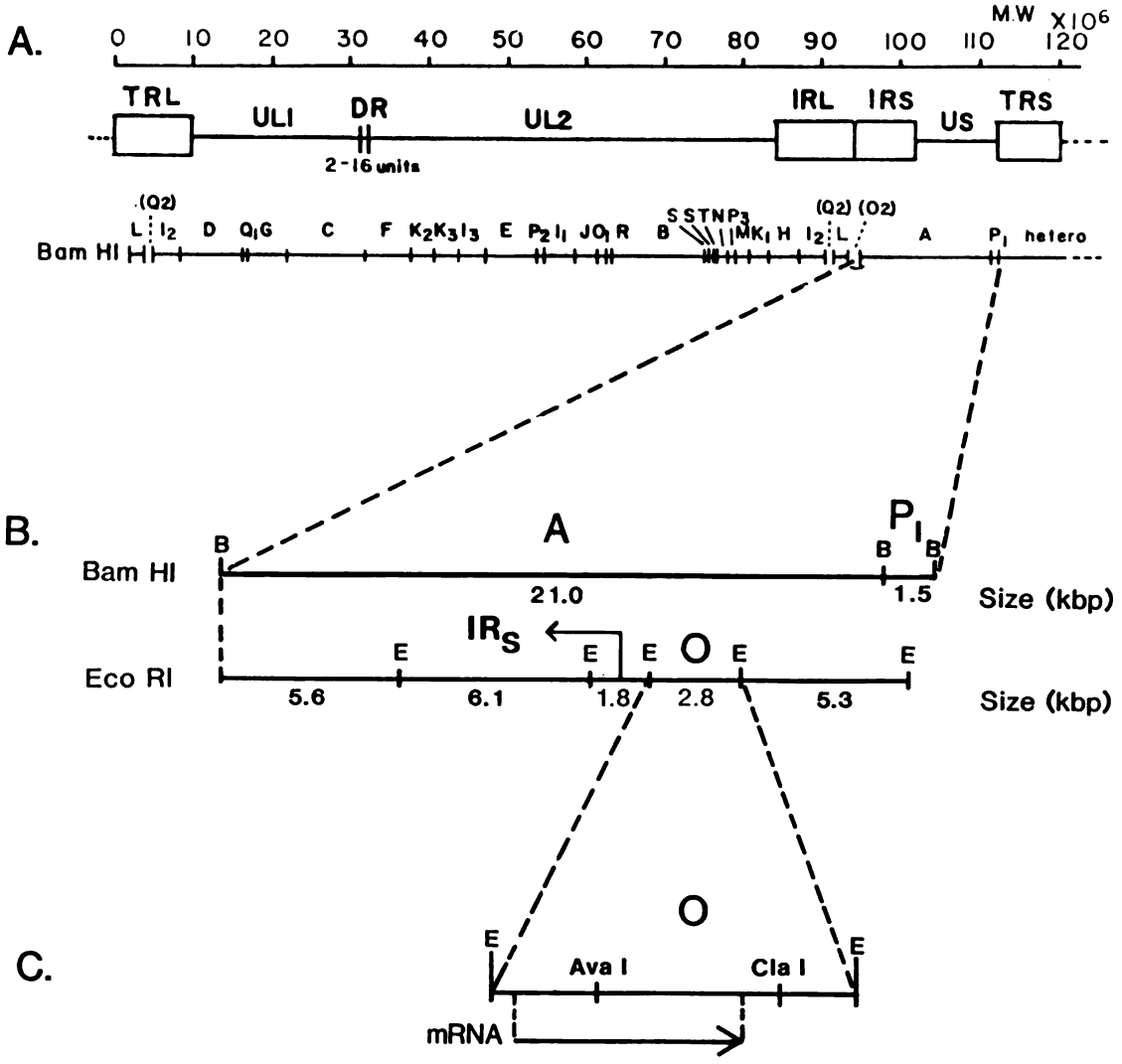


FIGURE 2. Northern blot analysis of the 1.9 kb Eco RI-O transcript from MDV-infected cells.

Thirty μg per lane of whole RNA from mock (lanes 1,5,8)- or MDV (lanes 2,3,4,6,7,9,10)-infected cells treated with (lanes 7,10) or without (lanes 1-6,8,9) PAA was fractionated on a 2.2 M formaldehyde-1.2% agarose gel, blotted onto nitrocellulose and hybridized with an Eco RI-O DNA probe (lanes 1,2,5,6,7), an EcoRI-O RNA probe (lanes 3,4) or an MDV gp57-65 PAA-sensitive control probe (lanes 8,9,10). Sizes were determined by ethidium bromide staining of an RNA ladder (BRL).

NORTHERN BLOT ANALYSES OF THE 1.9 kb EcoRI-O TRANSCRIPT FROM MDV-INFECTED CELLS

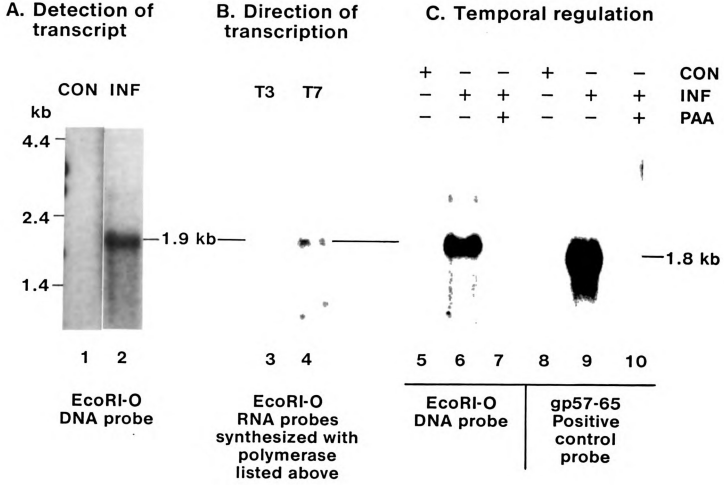
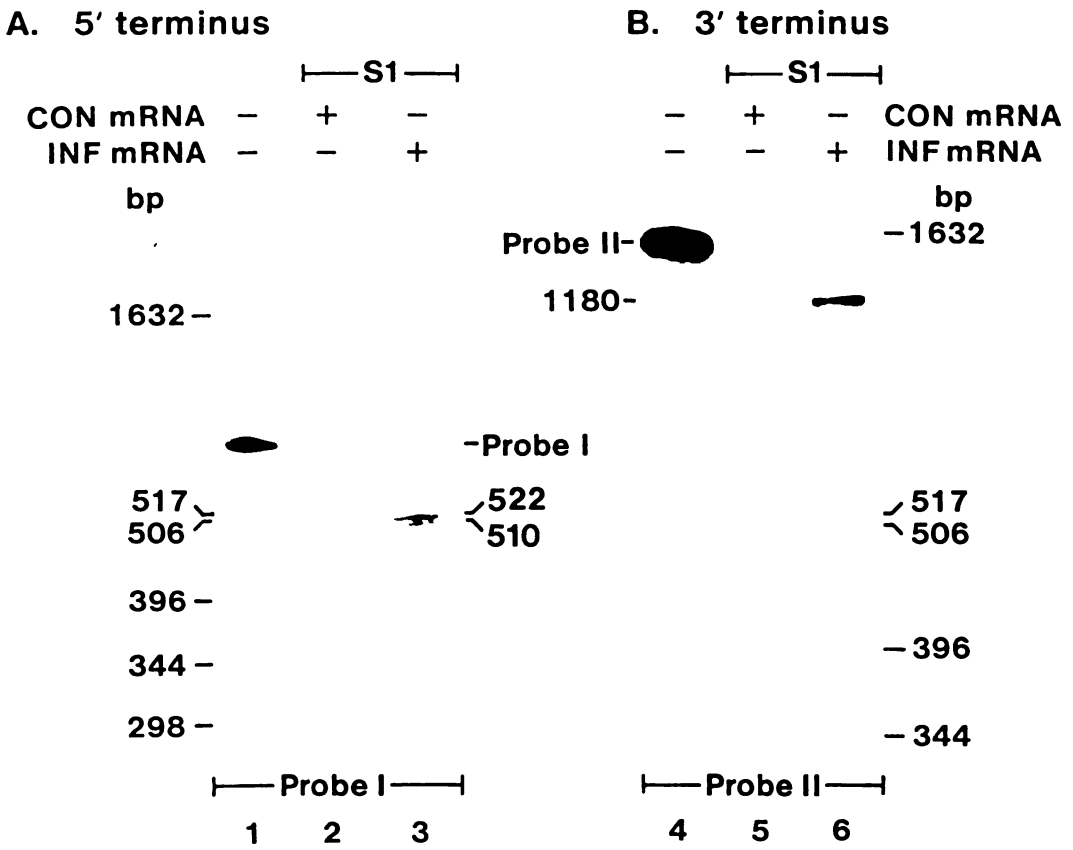


FIGURE 3. S1 nuclease protection analysis of the 1.9 kb Eco RI-O transcript.

A,B) Strand-specific probes described in Figure 3C below were labeled at their 5' (lanes 1-3)- or 3' (lanes 4-6) ends and hybridized with 1 μg of poly $(A)^+$-selected mRNA from mock (lanes 2,5)- or MDV (lanes 3,6)-infected cells. The RNA-DNA hybrids were digested with S1 endonuclease and their reaction products electrophoresed on 6% alkaline agarose gels next to the intact probes (lanes 1,3). An end-labeled pBR322/HinfI digest was included as a molecular weight standard.

C) The results of Figures 3A and 3B are interpreted in relation to the published nucleotide sequence which spans this region (Brunovskis and Velicer, 1992a). Outlined is a description of probes I and II, localization of the S1-protected mRNA, and locations of TATA and polyadenylation signals relative to the US1 and US10 ORFs.

S1 NUCLEASE PROTECTION ANALYSIS OF THE 1.9 kb EcoR1-O TRANSCRIPT



nucleotides were detected (Figure 3A, lane 3). When the 3'-labeled 1.5 kb AvaI-ClaI probe (Probe II, Figure 3C) was hybridized a single fragment of 1,180 nucleotides was protected from S1 nuclease digestion (Figure 3B, lane 6). Together with a poly $(A)^+$ tail of about 200 nucleotides, the combined sizes of the protected fragments are consistent with the 1.9 kb transcript identified above.

Having mapped this transcript, the corresponding DNA sequence was determined. Although it was initially thought that the 1.9 kb abundantly expressed late transcript was likely to encode an alphaherpesvirus-related glycoprotein gene homolog, this was not the case. Nucleotide sequence analysis identified two non-overlapping open reading frames (ORFs) within the region encoded by this transcript; these correspond to the MDV homologs of HSV-1 US1 (ICP22) and US10, a putative virion protein (Brunovskis and Velicer, 1992a; McGeoch et al., 1988). S1 nuclease protection analysis mapped the transcriptional initiation site to a site approximately 20 nucleotides downstream of a TATA consensus sequence, TATATAATA located 72 nucleotides upstream of the putative initiation codon of MDV US1 (Figure 3C; Brunovskis and Velicer, 1992a). The 3' end was localized approximately 91-122 nucleotides downstream of consensus polyadenylation- (AATAAA) and GT-rich (GTGTTGT) sequences located 92 and 61 nucleotides downstream, respectively, of the US10 termination codon (Figure 1C; Brunovskis and Velicer, 1992a). Together, these results are consistent with previously established HSV-1 transcription patterns, characterized by initiation from gene-specific promoters located upstream of, and close to, the start of each ORF, often terminating at common sites adjacent to downstream ORFs (McGeoch, 1991). On the basis of these criteria, and a consideration of the scanning model for translation (Kozak, 1989), MDV's ICP22-

homologous polypeptide would be expected to be expressed from the 1.9 kb transcript.

Identification of the MDV US1 gene product, pp27,24. To further analyze MDV US1 expression at the polypeptide level, polyclonal, monospecific antisera were generated by immunization of rabbits with bacterially synthesized *trpE*-US1 fusion proteins (see Materials and Methods). Preliminary experiments utilized this antiserum (RαUS1) to immunoprecipitate a 27-kDa MDV-specific polypeptide from infected DEF cells labeled with [^{35}S]-methionine at late times post-infection (72 hr) when significant cytopathic effects were observed (data not shown). The specificity of RαUS1 was demonstrated by an antibody blocking experiment (Figure 4A). Preincubation of RαUS1 with *trpE* gene product alone had no effect on the precipitation of the 27-kDa polypeptide (lane 2); in contrast, preincubation of RαUS1 with *trpE*-US1 gene product prevented the precipitation of this 27-kDa polypeptide. (lane 3). To determine whether pp27 is modified by phosphorylation like its HSV-1 counterpart (Wilcox et al., 1980), immunoprecipitation analysis of [^{32}P]-orthophosphate labeled cells was performed using RaUS1. These studies showed that the 27-kDa polypeptide (heretofore designated as pp27) could incorporate radioactive label from both both ^{32}P- and ^{35}S-labeled cells (Figure 4A, lanes 5,7). Interestingly, inclusion of the additional protease inhibitors TPCK, TLCK, and aprotinin, enhanced the recovery of an additional lower abundance polypeptide, pp24.

Serotype specificity of pp27,24. Herpesvirus of turkeys is a naturally apathogenic vaccine strain (serotype 3) that is highly related to MDV (Calnek and Witter, 1991). Although one study failed to demonstrate homology between the U_S regions of these two serotypes (Igarashi et al., 1987), recent nucleotide sequence analysis indicates that HVT's U_S region does contain a colinear assortment of alphaherpesvirus-related genes (M. Wild, pers. comm.). Since

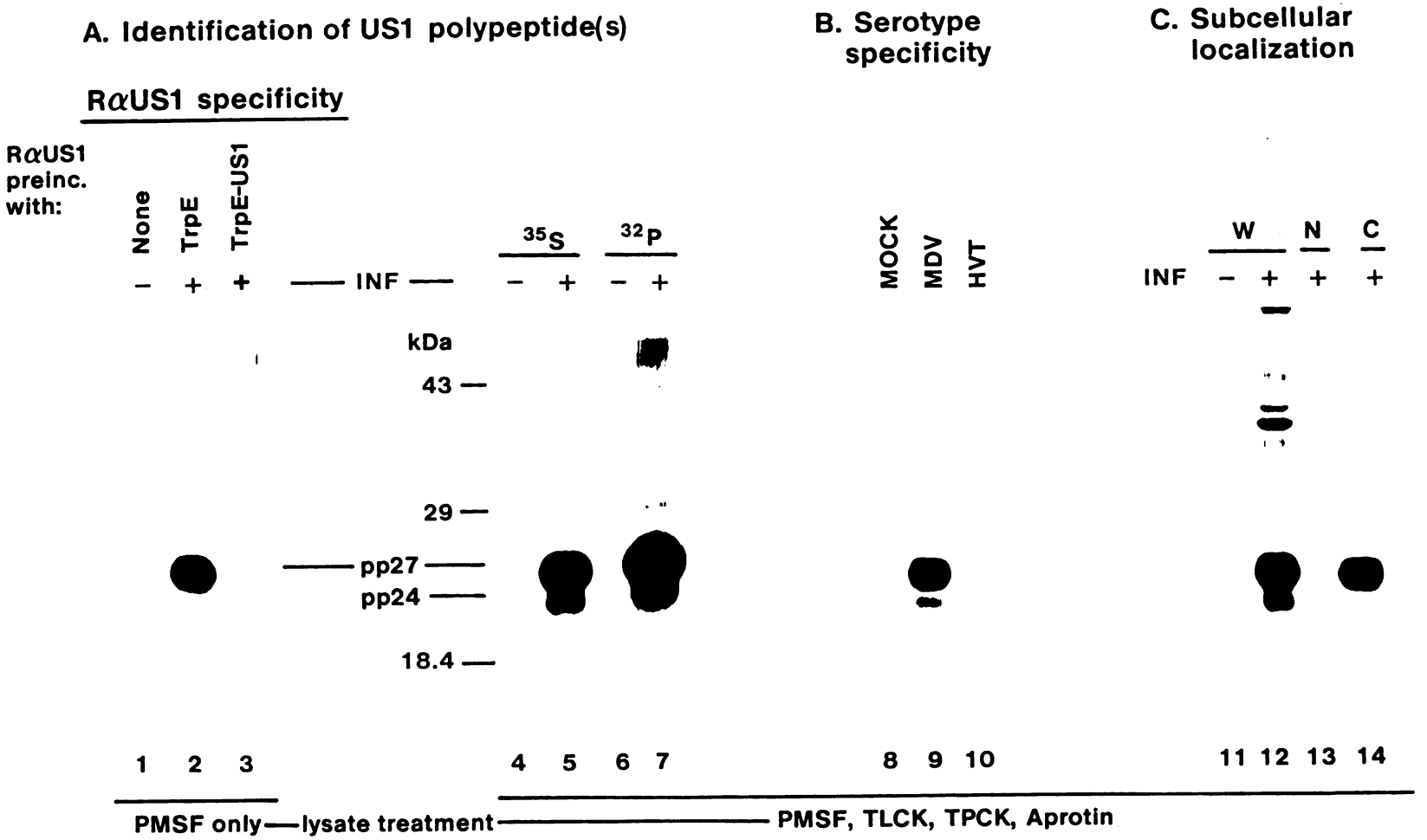
FIGURE 4. Identification, characterization and subcellular localization of the MDV US1 polypeptide, pp27,24.

A) RαUS1 specificity and phosphorylation of US1 polypeptide. Mock (lane 1,4,6)- or MDV (lanes 2,3,5,7)-infected DEF cells were labeled with [^{35}S]-methionine (lanes 1-5) or [^{32}P]-orthophosphate (lanes 6,7) and immunoprecipitated with RαUS1 (lanes 1,4-7) or with RαUS1 preincubated with either solubilized *trpE* (lane 2) or *trpE*-US1 (lane 3). Lysates were treated with the protease inhibitors, PMSF (lanes 1-3) or PMSF plus TLCK, TPCK, and aprotinin (lanes 4-14).

B) Serotype specificity. Cells from mock (lane 8)-, MDV (lane 9)- or HVT (lanes 10)-infected cells were labeled with [^{35}S]-methionine and analyzed by immunoprecipitation analysis with RαUS1.

C) Subcellular localization of pp27,24. RαUS1 was used to immunoprecipitate pp27,24 from mock (lane 11)- or MDV (lanes 12-14)-infected whole cell (lanes 11,12)-, nuclear (lanes 13)- or cytoplasmic (lane 14) fractions.

IDENTIFICATION, CHARACTERIZATION AND SUBCELLULAR LOCALIZATION OF THE MDV US1 POLYPEPTIDE, pp27, 24



HVT proteins share many immunologically cross-reactive epitopes with MDV, it was of interest to examine whether RαUS1 could precipitate an HVT ICP22 counterpart. Immunoprecipitation analysis of HVT-infected cell lysates failed to identify an HVT US1 counterpart under standard conditions (Figure 4B, lane 10) or following immunoprecipitation analysis of lysates previously denatured by boiling in 1% SDS/2-ME (data not shown).

Subcellular localization of pp27,24. Previous investigators have indicated that a significant fraction of HSV-1 ICP22 is located in the nucleus (Fenwick et al., 1978, 1980; Hay and Hay, 1980; Wilcox et al., 1980), possibly accounting for an ability to affect gene expression (Jackers et al., 1992; Jacquemont et al., 1984; Sears et al., 1985). In contrast to HSV-1, immunoprecipitation analysis of nuclear and cytosolic fractions from MDV-infected cells indicate that pp27,24 is a cytosolic protein, at least in DEF cells (Figure 4D, lane 14). This result was not a reflection of inappropriate fractionation, since a nuclear antigen control serum was positive only for the nuclear fraction (data not shown). Despite saving and testing all fractions recovered, most of the pp24 (as well as non-specifically bound polypeptides identified in the whole cell fraction) was lost during the fractionation (Figure 4C, lane 14). A minor proportion of pp24 was identified upon longer exposures. Considering the difficulties in detecting this polypeptide in the absence of additional protease inhibitors (except after long exposures), loss of pp24 following fractionation suggests that it is highly susceptible to spontaneous- or proteolytic degradation.

Temporal regulation of pp27,24. To confirm the PAA-sensitivity of pp27,24 expression at the polypeptide level, immunoprecipitation tests were carried out using lysates from PAA-treated cells. Similar to the results obtained by Northern blot analysis (Figure 2C), pp27,24 expression was substantially inhibited in PAA-treated cultures (Figure 5A, lanes 1 and 2); low levels of pp27,24 could be

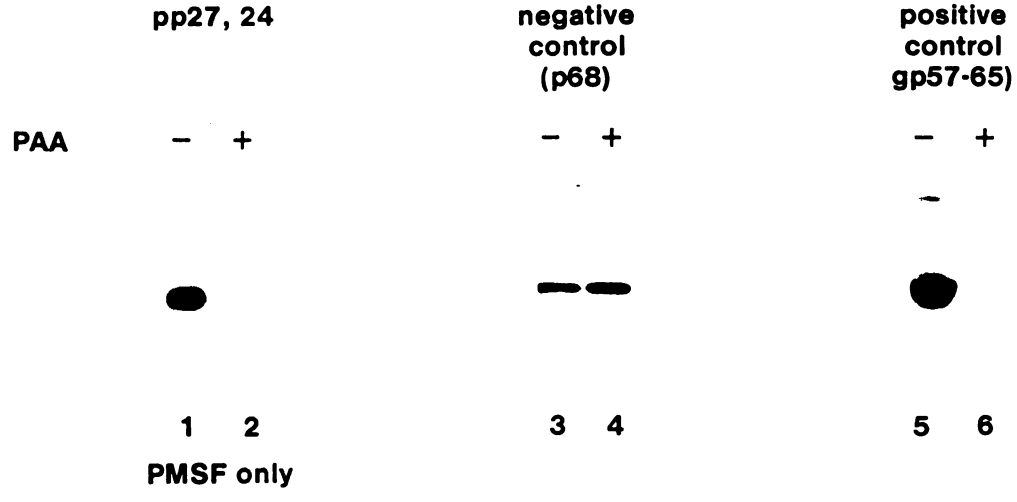
FIGURE 5. Temporal regulation of the MDV US1 homolog.

A) pp27,24 is regulated as a late protein. Immunoprecipitation analysis of infected-cell lysates (treated with PMSF only) from untreated (lanes 1,3,5) or PAA-treated (lanes 2,4,6) cells was with RαUS1 (lanes 1,2) or the two control sera, RαUS3/89-176 (lanes 3,4), an MDV US3/PK-directed sera cross-reactive with a 68-kDa cellular protein (Brunovskis and Velicer, 1992b), and ICS (immune chicken serum; lanes 5,6), an antiserum used to precipitate the secreted glycoprotein, gp57-65, from infected cell culture medium.

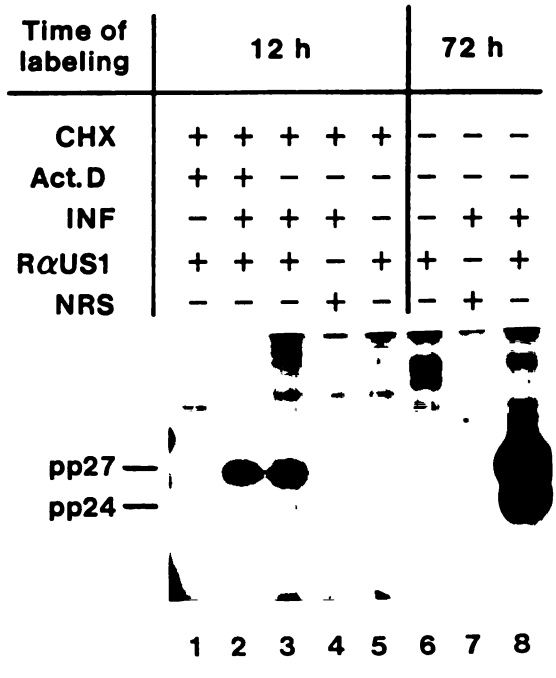
B) pp27,24 expression is not induced at the immediate-early level. Mock (lanes 1,5)- or MDV (lanes 2-4,7,8)-infected cells were pulse-labeled for 1 hr in the presence of [^{35}S]-methionine and actinomycin D (Act. D) after removal of cycloheximide (CHX) present at 0-12 hr postinfection with (lanes 1,2) or without (lanes 3-8) Act. D. Lysates from these cells were subjected to immunoprecipitation analysis with RαUS1 (lanes 1,2,3,5,6,8) or normal rabbit serum (NRS; lanes 4). Control lysates from mock (lane 6)- or MDV (lanes 7,8)-infected cells labeled at 72 hr postinfection were subjected to immunoprecipitation with RαUS1 (lanes 6,8) or NRS (lane 7).

TEMPORAL REGULATION OF THE MDV US1 HOMOLOG

A. pp27, 24 is regulated as a late gene



B. pp27, 24 expression is not induced at the immediate-early level



detected only following long exposure times (data not shown). Expression of a PAA-insensitive cellular control protein (p68, Brunovskis and Velicer, 1992b) was unaffected by PAA-treatment (lanes 3,4) in contrast to the late control, MDV gp57-65 (lanes 5,6). These results are consistent with earlier Northern blot analysis suggesting late gene regulation of MDV US1. The presence of pp27,24 in PAA-treated cells following long exposures is consistent with either gamma-1 regulation or the presence of gamma-2 transcripts in the infected cell inoculum that are still translatable 72 hr postinfection.

In light of the slow infection kinetics of MDV, and other US1 homologs (EHV-1, BHV-1) being regulated at multiple levels (i.e. IE, E, and/or L times postinfection; Holden et al., 1992; M. Schwyzer, pers. comm.), we next examined whether MDV US1 expression may be additionally induced in the presence of cycloheximide (CHX), an inhibitor of translation. Initially, Northern blot analysis appeared to support this possibility (data not shown). Furthermore, pp27,24 was consistently detected by immunoprecipitation/SDS-PAGE analysis of DEF cells infected for 12 hrs in the presence of CHX, followed by a 1 hr pulse with [^{35}S]-methionine in the absence of CHX and presence of actinomycin D (Act. D). The latter is known to inhibit further transcription. Assessment of IE gene induction was complicated by the highly cell-associated culture conditions for MDV growth. Such conditions could potentially account for the detection and translatability of abundantly expressed late transcripts in the presence of CHX- or PAA. Consistent with this possibility was the observation that comparable pp27,24 detection levels required over 10-fold longer X-ray exposure times for immunoprecipitates assayed under IE vs. L conditions. To determine whether these polypeptides represent low abundance IE products, a CHX block-release experiment was performed in which infected cells were treated with CHX in the presence or absence of Act. D. Under these conditions an IE gene would be

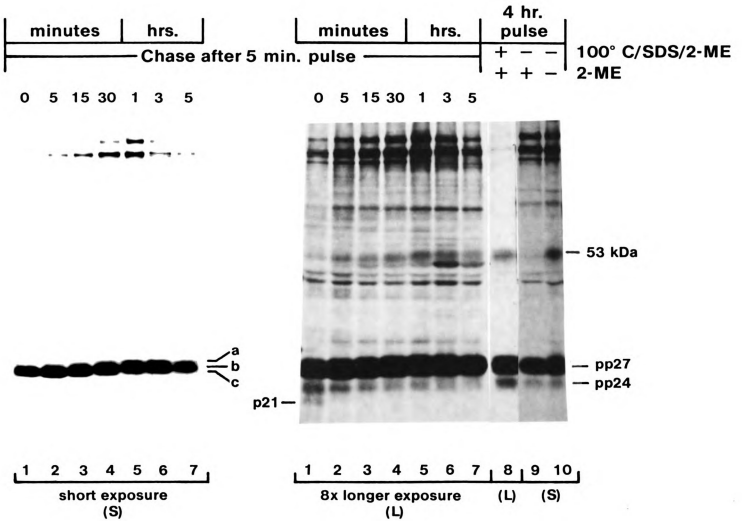
expected to produce higher levels of protein in cells not treated with Act. D; polypeptide levels corresponding to late transcripts present in the infected cell inoculum would be unaffected by the presence of Act. D. As shown in Figure 5B (compare lanes 2 and 3), the latter turned out to be the case. This result indicates that MDV US1 expression is not inducible under immediate-early conditions.

Posttranslational processing of pp27,24. Processing kinetics of pp27,24 were studied at various chase times following an initial 5 min pulse with [^{35}S]-methionine (Figure 6). Three closely migrating polypeptides, designated as pp27a, pp27b, and pp27c (Figure 6, short exposure) were identified. A processing pattern was observed whereby pp27c was sequentially processed to pp27b and/or pp27a (Figure 6, short exposure). A similar processing pattern, characterized by the sequential formation of higher Mr products following longer chase periods, was observed for polypeptides migrating below (24-kDa) or above (53-kDa) the major products referred to above (Figure 6, longer exposure). These polypeptides were similarly observed in 4 hr labeled cells, particularly with longer exposure times (Figure 6, lane 8). All three groups of polypeptides were unique to infected cells and were similarly observed in cell cultures pulse-labeled with [^{32}P]-orthophosphate (data not shown); however, the latter experiments did not allow for the resolution or discrimination between the three distinct polypeptides observed in [^{35}S]-methionine labeled cells using shorter exposures (Figure 6; data not shown). These additional phosphoproteins do not appear to coprecipitate with pp27, since their presence was similarly observed in cell lysates boiled in 1% SDS and 2-ME prior to the addition of RαUS1 (Figure 6, lane 8). Electrophoresis under non-reducing conditions led to a marked increase in the level of the 53-kDa polypeptide

FIGURE 6. pp27,24 processing kinetics and characterization.

Infected cells were pulse-labeled for 5 min with [^{35}S]-methionine and chased in the presence of excess methionine for the number of minutes or hours indicated (lanes 1-7). Lysates from cells labeled for 4 hr were boiled for 5 min in 1% SDS and 1% 2-mercaptoethanol (2-ME) prior to immunoprecipitation analysis with RαUS1 (lane 8); non-pre-treated lysates were analyzed with RαUS1 by electrophoresis under reducing (lane 9)- or non-reducing (lane 10) conditions.

pp27, 24 PROCESSING KINETICS AND CHARACTERIZATION



(Figure 6, compare lanes 9,10), raising the possibility that pp27,24 forms disulfide-linked dimers under native conditions.

Longer exposure times revealed the presence of a unique polypeptide with increased mobility (21-kDa) that was only detected in the absence of a chase (lane 8, longer exposure). This polypeptide was not detected in a similar experiment conducted with [^{32}-P]-orthophosphate (data not shown). The 21-kDa size of this polypeptide is consistent with the predicted size of the US1 polypeptide based on its primary amino acid sequence (Brunovskis and Velicer, 1992a). These data are consistent with the suggestion that p21 is the primary precusor polypeptide of US1, which is rapidly phosphorylated shortly after translation resulting in closely migrating phosphoproteins of 27-kDa, possibly leading to the formation of 53-kDa dimers. Demonstration of a true precusor-product relationship between p21 and pp27,24 will require the use of shorter pulse (1 min) and chase (0 - 10 min) intervals.

DISCUSSION

The salient features of our results and their implications are as follows:

(i) Unlike its immediate-early HSV-1 counterpart, MDV's US1 homolog is regulated as a late gene. PAA-sensitive expression was observed at both transcriptional- and translational levels. This result highlights significant differences in the regulation of this alphaherpesvirus-specific gene. PRV has only one IE gene (ICP4 homolog; Cheung, 1989); failing to find immediate-early regulation of PRV US1 was therefore not a surprise (Zhang and Leader, 1990b). The bovine- and equine ICP22 homologs are apparently regulated at *two* different levels. BHV-1 ICP22 is an abundantly expressed 55-kDa phosphoprotein, regulated at both IE and late times (M. Schwyzer, pers. comm.); EHV-1 Kentucky A strain ICP22 is expressed from both early and late promotors, resulting in two different transcripts potentially coding for proteins of 293- or 469 aa, respectively (Holden et al., 1992). These recent observations appear consistent with a much earlier prediction (Honess, 1984) invoking the recombination/isomerization process to account for the possible dislocation of normal regulatory elements from genes (or their homologs) with transcripts crossing the boundaries of the repeat-unique junctions (such as HSV US1) thereby altering their normal gene regulation. While MDV's US1 homolog is located entirely in its U_S region, PRV, EHV-1, BHV-1, and VZV US1 genes are found as two copies in the adjoining repeats.

(ii) MDV US1 appears to be transcribed from a 1.9 kb mRNA initiating near the US1 initiation codon and terminating just downstream of an adjacent ORF coding for the MDV homolog of HSV-1 US10. This is based on results from Northern blot- and nuclease S1 protection analyses. The lengths of the protected fragments were consistent with the length of the 1.9 kb transcript

identified by Northern blot analysis. Based on the scanning model for translation (Kozak, 1989) and an analysis of herpesvirus transcription characteristics (McGeoch, 1991), it was concluded that pp27,24 is most likely translated from the 1.9 kb transcript, which contains coding sequences for both US1 and US10. Smaller, US10-specific transcripts could not be detected in the same cells found to express a 24-kDa phosphorylated US10-related phosphoprotein detectable with *trpE*-US10-directed antisera (Brunovskis and Velicer, 1992b). This suggests that the 1.9 kb US1 transcript may represent an unusual bicistronic mRNA directing the translation of two independent, non-overlapping open reading frames. A similar proposal has recently been reported to account for the expression of two PRV U_S region polypeptides from a common transcript (Kost et al., 1989). Confirmation of this point awaits future experiments testing whether mRNAs hybrid-selected with a US1-specific probe can be translated into reaction products that can be precipitated by both US1- and US10-specific antisera.

(iii) In sharp contrast to the 68-kDa nuclear product which corresponds to HSV-1 ICP22, MDV's ICP22 counterpart was characterized as a much smaller 27-kDa cytosolic product (Figure 4C). Not only is HSV-1 ICP22 a predominantly nuclear protein (Fenwick et al., 1978; Fenwick et al., 1980), it is reported to associate with chromatin (Hay and Hay, 1980). It will be of interest to determine whether the cytoplasmic localization of pp27,24 and/or differences in its temporal regulation adversely affect its ability to regulate gene expression. Sequestration in the cytoplasm could account for slow growth of MDV in cell cultures and/or account for its inability to promote fully productive infections in vitro. Changes in the subcellar localization of pp27,24 in different cell types could lead to different patterns of gene expression. A recent report has indicated that the redistribution of EBV BZLF1 from the cytoplasm to nucleus

correlates with increasing differentiation of epithelial cells in oral hairy leukoplakia and a shift to a lytic pattern of gene expression characterized by expression of the virus capsid antigen, BcLF1 (Becker et al., 1991).

(iv) HVT appears to lack an ICP22 homolog. Because of the pathogenicity associated with HSV-1 US1 (Meignier et al., 1988), such a finding may be of relevance to HVT's apathogenic nature.

(v) Like ICP22, pp27,24 was found to be phosphorylated (Figure 2). Pulse-chase studies indicated that pp27,24 exists in at least three distinct forms. This is identical to results obtained with HSV-1 ICP22 (Fenwick et al., 1980) and ICP4 (Wilcox et al., 1980). In the former case, ICP22 was found to be modified by phosphorylation in at least two steps; the larger of the three forms appeared to depend on the expression of a later class product. In the case of MDV, pp27b represents the major species identified. Except for ICP47, all HSV-1 IE proteins exhibit phosphorylation patterns that are accompanied by apparent increases in molecular weight (Everett, 1987). HSV-1 ICP22 has been reported to be the most extensively phosphorylated protein of a group that includes ICP0, ICP4, and ICP27 (Ackermann, 1985). The extent of phosphorylation has been shown to modulate differential recognition of leader sequences of different temporal classes of HSV promoters (Papavassiliou et al., 1991). Aside from MDV and HSV-1, nothing more is known concerning phosphorylation of other ICP22 homologs.

It has been reported that phosphates cycle on and off of HSV-1 ICP22 (Wilcox et al., 1980). Our pulse chase studies failed to support this view. Phosphate remained stably associated with pp27,24 through chase periods as long as 12 hours (data not shown). The previous study (Wilcox et al., 1980) interpreted the loss of phosphate following a 15 hr PO_4-free chase and its subsequent reincorporation during a 2 hr pulse beginning at 16 hr postinfection

as evidence for the cycling of phosphate. Rather than representing rephosphorylation of preexisting dephosphorylated ICP22 at 16-18 hr post-infection, newly incorporated label may instead be derived from newly translated transcripts. This is suggested by earlier HSV-1 studies demonstrating the continued synthesis of translatable ICP22 transcripts late after infection (Anderson et al., 1980). Our own results indicated that MDV's US1 transcript is a stable, long half-life transcript translatable from infected cell inocula maintained in culture for at least 12 hr.

Our attempts to identify a potential IE counterpart of pp27,24 point to fundamental difficulties associated with temporal gene regulation studies conducted with cell-associated viruses like MDV. Due to the inability of creating high-titer stocks of cell-free virus (e.g. in feather follicle epithelial cells), temporal gene regulation studies in the MDV system generally depend on passing infected cells onto uninfected monolayers. As noted above, late US1 transcripts present in the infected cell inoculum can maintain their stability and translatability (for at least 12 hours) following passage onto uninfected monolayers treated with CHX. The combined use of CHX, with and without actinomycin D, provides a useful approach for temporal regulation studies involving strictly cell-associated viruses. Failure to address these potential difficulties may have led others (Schat et al., 1989) to incorrectly identify as "immediate-early" a similarly-sized 1.7 kb MDV transcript, which mapped to the same 2.8 kb EcoRI subfragment as US1 (Figure 1).

Little is known concerning the role of ICP22-related polypeptides. HSV-1 US1 mutants are known to grow poorly in some cell lines but not others (Sears et al., 1985); this has led to the suggestion that certain cells can complement US1 mutants by providing an ICP22-related host function. It should be pointed out that despite the reports characterizing ICP22 as 'nonessential' for growth in

cell culture, the mutants employed still maintain the ability to express the NH_2-terminal 200 aa of HSV-1's ICP22 (Ackermann et al., 1985; Post and Roizman, 1981; McGeoch et al., 1985). This results in the expression of a 33.7-kDa phosphoprotein in HEp-2 cells and at least three distinct 33.7-39-kDa phosphoproteins in BHK cells (Ackermann et al., 1985). Like the full-length ICP22, the truncated polypeptides were similarly phosphorylated and localized to the nucleus. These truncated derivatives may have residual function for cell culture growth in view of a report that oligo(nucleoside methylphosphonate) dodecamer derivatives complementary to the splice junction of the ICP22 mRNA-4 effected a 98% decrease in HSV-1 titers with little, if any, deletarious effects on host-cell macromolecular metabolism (Kulka et al., 1989).

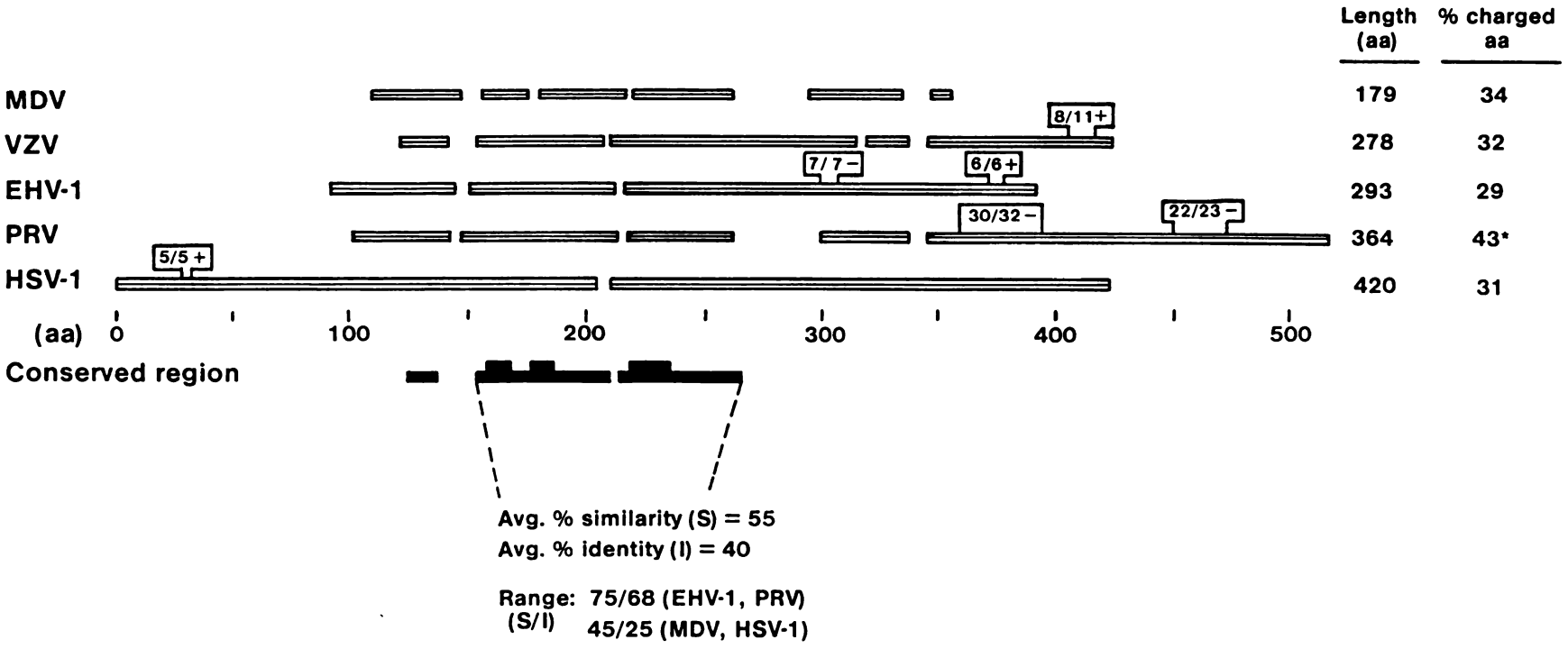
It is tempting to speculate that the supplementary essential, cell tropism-determining nature of ICP22 is attributed to its role as a transcription factor, whose activity is critical for growth in certain cell types, but not others. ICP22 homologs are noted for containing distinctive charge configurations (Karlin et al., 1989; Zhang and Leader, 1990) characteristic of transcription factors (Figure 7; Brendel and Karlin, 1989). HSV-1 ICP22 mutants have been previously characterized as being defective for late gene expression (Sears et al., 1985). The VZV ICP22 homolog encoded by gene 63 has recently been reported to repress the IE promoter of its ICP4 homolog, but stimulate the E promoter of its TK homolog (Jackers et al., 1992). On the other hand, the bovine herpesvirus type 1 (BHV-1) ICP22 homolog has recently been found to encode an abundantly expressed 55-kDa phosphoprotein inhibiting target promoters of all kinetic classes tested (M. Schwyzer, pers. comm.).

One of the most interesting aspects about ICP22-related homologs is their profound divergence in sequence conservation and size (Figure 7). They can be as small as 179 aa (MDV) or as large as 420 aa (HSV-1). Conserved

FIGURE 7. Homology and divergence of US1 (ICP22) homologs.

Predicted amino acid sequences for the US1 homologs of MDV (Brunovskis and Velicer, 1992a), VZV (Davison and Scott, 1986), EHV-1 (Holden et al., 1992), PRV (Zhang and Leader, 1990), and HSV-1 (McGeoch et al., 1985) were aligned with the UWGCG program GAP. Aligned (lined boxes) and conserved (filled boxes) regions are displayed whereby the lower- and upper-case filled areas define areas in which at least 3/5 homologs exhibit 5-7 or greater than 7 identities, respectively over a sliding window of 10 residues. Included are the lengths of each homolog, their % charged residue content, and % similarity/identity analyses of the conserved region.

HOMOLOGY AND DIVERGENCE OF US1(ICP22) HOMOLOGS



*99/137 (72%) aa at carboxy-end are aspartic-(D) or glutamic acid (E)

sequences are primarily limited to a 103-aa region which fails to exhibit any obvious structure-function relationships (Figure 7). Pairwise comparisons between ICP22 homologs in this region result in average similarities and identities of 55% and 40%, respectively. However, even in this conserved region, the level of homology can range from as low as 45% similarity/25% identity (MDV, HSV-1) to 75% similarity/68% identity (EHV-1, PRV). Perhaps this region allows for the creation of unique protein-protein interactions responsible for differential gene expression depending on the cell type. It is possible that ICP22-related homologs exhibit a basic level of functional conservation conferring interactions with itself and/or transcription factors. ICP4 has previously been shown to homodimerize (Metzler and Wilcox, 1985) and operationally substitute for the cellular transcriptional factor Sp1 in promoting the efficient expression of the viral thymidine kinase gene. Our preliminary results suggest that pp27,24 can also homodimerize. Further work will be necessary to confirm this possibility and to determine the significance of its cytosolic localization in relation to MDV growth.

REFERENCES

Ackermann, M., M. Sarmiento, and B. Roizman. 1985. Application of antibody to synthetic peptides for characterization of the intact and truncated α22 protein specified by herpes simplex virus 1 and the R325 α22⁻ deletion mutant. J. Virol. **56**:207-215.

Anderson, K. P., R. C. Costa, L. E. Holland, and E. K. Wagner. 1980. Characterization of herpes simplex virus type 1 RNA present in the absence of de novo protein synthesis. J. Virol. **34**:9-27.

Becker, J., U. Leser, M. Marschall, A. Langford, W. Jilg, H. Gelderblom, P. Reichart, and H. Wolf. 1991. Expression of proteins encoded by Epstein-Barr virus trans-activator genes depends on the differentiation of epithelial cells in oral hairy leukoplakia. Proc. Natl. Acad. Sci. USA. **88**:8332-8336.

Binns, M. M., and N.L.J. Ross. 1989. Nucleotide sequence of the Marek's disease virus (MDV) RB-1B A antigen gene and the identification of the MDV A antigen as the herpes simplex virus-1 glycoprotein C homologue. Virus Res. **12**:371-382.

Brendel, V. and S. Karlin. 1989. Association of charge clusters with functional domains of cellular transcription factors. Proc. Natl. Acad. Sci. USA **86**:5698-5702.

Brunovskis, P. and L. F. Velicer. 1992a. Structural characterization of the Marek's disease virus (MDV) unique short region: Presence of alphaherpesvirus-homologous, fowlpox virus-homologous and MDV-specific genes. Submitted for publication.

Brunovskis, P. and L. F. Velicer. 1992b. Analysis of Marek's disease virus unique short region polypeptide expression with antisera to inducible, bacterially expressed fusion proteins. Manuscript in preparation.

Buckmaster, A. E., S. D. Scott, M. J. Sanderson, M.E.G. Boursnell, N.L.J. Ross, and M. M. Binns. 1988. Gene sequence and mapping data from Marek's disease virus and herpesvirus of turkeys: Implication for herpesvirus classification. J. Gen. Virol. **69**:2033-2042.

Calnek, B. W. and R. L. Witter. 1991. Marek's disease, p. 342-385. *In* B. W. Calnek (ed.), Diseases of poultry, Iowa State University Press.

Chen, X., P.J.A. Sondermeijer, and L. F. Velicer. 1992. Identification of a unique Marek's disease virus gene which encodes a 38-kilodalton phosphoprotein and is expressed in both lytically infected cells and latently infected lymphoblastoid tumor cells. J. Virol. **66**:85-94.

Cheung, A. K. 1989. DNA nucleotide sequence analysis of the immediate-early gene of pseudorabies virus. 1989. Nucl. Acids Res. **17**:4637-4646.

Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by guanidinium-thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**:156-159.

Croen, K.D., J. M. Ostrove, L. J. Dragovic, and S. E. Straus. 1988. Patterns of gene expression are different for varicella-zoster and herpes simplex viruses. Proc. Natl. Acad. Sci. USA **85**:9773-9777.

Corden, B. Wasylyk, A. Buchwalder, P. Sassone-Corsi, C. Kedinger, and P. Chambon. 1980. Promoter sequences of eukaryotic protein-coding genes. Science **209**:1406-1414.

Coussens, P. M., and L. F. Velicer. 1988. Structure and complete nucleotide sequence of the Marek's disease herpesvirus gp57-65 gene. J. Virol. **62**:2373-2379.

Cullinane, A. A., F. J. Rixon, and A. J. Davison. 1988. Characterization of the genome of equine herpesvirus 1 subtype 2. J. Gen. Virol. **69**:1575-1590.

Davison, A. J., and N. M. Wilkie. 1983. Location and orientation of homologous sequences in the genomes of five herpesviruses. J. Gen. Virol. **64**:1927-1942.

Davison, A. J., and D. J. McGeoch. 1986. Evolutionary comparisons of the S segments in the genomes of herpes simplex virus type 1 and varicella-zoster virus. J. Gen. Virol. **67**:597-611.

Davison, A. J., and J. E. Scott. 1986. The complete DNA sequence of varicella-zoster virus. J. Gen. Virol. **76**:1759-1816.

Davison, A. J., and P. Taylor. 1987. Genetic relations between varicella-zoster virus and Epstein-Barr virus. J. Gen. Virol. **68**:1067-1079.

de Wind, N., A. Zijderveld, K. Glazenburg, A. Gielkens, and A. Berns. 1990. Linker insertion mutagenesis of herpesviruses: inactivation of single genes within the U_S region of pseudorabies virus. J. Virol. **64**:4691-4696.

Epstein, A. L. and B. Jacquemont. 1983. Virus polypeptide synthesis induced by herpes simplex virus in non-permissive rat XC cells. J. Gen. Virol. **64**:1499-1508.

Everett, R. D. 1984. *Trans* activation of transcription by herpes virus products: Requirement for two HSV-1 immediate-early polypeptides for maximal activity. EMBO J. **3**:3135-3141.

Everett, R. D. 1987. The regulation of transcription of viral and cellular genes by herpesvirus immediate-early gene products. Anticancer Research **7**:589-604.

Fenwick, M., M. Walker, and J. M. Petkevich. 1978. On the association of viral proteins with the nuclei of cells infected with herpes simplex virus. J. Gen. Virol. **39**:519-529.

Fenwick, M., M. Walker, and L. Marshall. 1980. Some characteristics of an early protein (ICP22) synthesized in cells infected with herpes simplex virus. J. Gen. Virol. **47**:333-341.

Fukuchi, K., M. Sudo, Y.-S. Lee, A. Tanaka, and M. Nonoyama. 1985. Structure of Marek's disease virus DNA: Detailed restriction enzyme map. J. Virol. **51**:102-109.

Hajjar, D. P. 1991. Viral pathogenesis of atherosclerosis: Impact of molecular mimicry and viral genes. Am J. Pathol. **139**:1195-1211.

Hay, R. T. and J. Hay. 1980. Properties of herpesvirus-induced "immediate-early" polypeptides. Virology **104**:230-234.

Hayes, M. K. and D. L. Rock. 1990. Identification of a novel bovine herpesvirus type 1 immediate-early infected cell protein. Arch. Virol. **112**:291-300.

Holden, V. R., R. R. Yalamanchili, R. N. Harty, and D. J. O'Callaghan. 1992. ICP22 homolog of equine herpesvirus 1: Expression from early and late promoters. J. Virol. **66**:664-673.

Honess, R. W. 1984. Herpes simplex and 'the herpes complex': diverse observations and a unifying hypothesis. J. Gen. Virol. **65**:2077-2107.

Igarashi, T., M. Takahashi, J. Donovan, J. Jessip, M. Smith, K. Hirai, A. Tanaka, and M. Nonoyama. 1987. Restriction enzyme map of herpesvirus of turkey DNA and its collinear relationship with Marek's disease virus DNA. Virology. **157**:351-358.

Isfort, R. J., L. Sithole, H.-J. Kung, and L. F. Velicer. 1986. Molecular characterization of Marek's disease herpesvirus B antigen. J. Virol. **59**:411-419.

Jackers, P., P. Defechereux, L. Baudoux, C. Lambert, M. Massaer, M.-P. Merville-Louis, B. Rentier, and J. Piette. 1992. Characterization of

regulatory functions of the varicella-zoster virus gene 63-encoded protein. J. Virol. 66:3899-3903.

Jacquemont, B., B. Verrier, A. L. Epstein, and I. Machuca. 1984. Expression of immediate-early genes in herpes simplex virus type 1-infected XC cells: Lack of ICP22 (68K) polypeptide. J. Gen. Virol. 65:1331-1340.

Karlin, S., B. E. Blaisdell, E. S. Mocarski, and V. Brendel. 1989. A method to identify distinctive charge configurations in protein sequences, with application to human herpesvirus polypeptides. J. Mol. Biol. 205:165-177.

Kimman, T. G., N. de Wind, N. Oei-Lie, J.M.A. Pol, A. J. Berns, and A.L.J. Gielkens. 1992. Contribution of single genes within the unique short region of Aujeszky's disease virus (suid herpesvirus type 1) to virulence, pathogenesis and immunogenicity. J. Gen Virol. 73:243-251.

Koerner, T. J., J. E. Hill, A. M. Myers, and A. Tzagoloff. 1991. High-expression vectors with multiple cloning sites for construction of *trpE* fusion genes: pATH vectors. Methods Enzymol. 194:477-490.

Kost, T. A., E. V. Jones, K. M. Smith, A. P. Reed, A. L. Brown, and T. J. Miller. 1989. Biological evaluation of glycoproteins mapping to two distinct mRNAs within the BamHI fragment 7 of pseudorabies virus: Expression of the coding regions by vaccinia virus. Virology. 171:365-376.

Kouzarides, T., A. T. Bankier, S. C. Satchwell, K. Weston, P. Tomlinson, and B. G. Barrell. 1987. Large-scale rearrangement of homologous regions in the genomes of HCMV and EBV. Virology 157:397-413.

Kozak, M. 1989. The scanning model for translation: An update. J. Cell Biol. 108:229-241.

Kulka, M., C. C. Smith, L. Aurelian, R. Fishelevich, K. Meade, P. Miller, and P.O.P. Ts'O. 1989. Site specificity of the inhibitory effects of oligo(nucleoside methylphosphonate)s complementary to the acceptor splice junction of herpes simplex virus type 1 immediate early mRNA 4. Proc. Natl. Acad. Sci. USA. 86:6868-6872.

Lee, L. F., K. Nazerian, S. S. Leinbach, J. M. Reno, and J. A. Boezi. 1976. Effect of phosphonoacetate on Marek's disease virus replication. J. Nat. Cancer Inst. 56:823-827.

Longnecker, R. and B. Roizman. 1986. Generation of an inverting herpes simplex virus 1 mutant lacking the L-S junction *a* sequences, an origin of DNA synthesis, and several genes including those specifying glycoprotein E and the a47 gene. J. Virol. 58:583-591.

Longnecker, R., S. Chatterjee, R. J. Whitley, and B. Roizman. 1987. Identification of a herpes simplex virus 1 glycoprotein gene within a gene cluster dispensable for growth in cell culture. Proc. Natl. Acad. Sci. USA 84:4303-4307.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

McGeoch, D. J., A. Dolan, S. Donald, and F. J. Rixon. 1985. Sequence determination and genetic content of the short unique region in the genome of herpes simplex virus type 1. J. Mol. Biol. 181:1-13.

McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor. 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. 69:1531-1574.

McGeoch, D. J. 1990. Evolutionary relationships of virion glycoprotein genes in the S regions of alphaherpesvirus genomes. J. Gen. Virol. 71:2361-2367.

McGeoch, D. J. 1991. Correlation between HSV-1 DNA sequence and viral transcription maps, p. 29-47. *In* E. K. Wagner (ed.), Herpesvirus transcription and its regulation. CRC Press, Inc., Boca Raton, Fla.

Meignier, B., R. Longnecker, P. Mavromara-Nazos, A. E. Sears, and B. Roizman. 1988. Virulence of and establishment of latency by genetically engineered deletion mutants of herpes simplex virus 1. Virology **162**:251-254.

Metzler, D. W. and K. W. Wilcox. 1985. Isolation of herpes simplex virus regulatory protein ICP4 as a homodimeric complex. J. Virol. **55**:329-337.

Murchie, M. J. and D. J. McGeoch. 1982. DNA sequence analysis of an immediate-early gene region of the herpes simplex virus type 1 genome (map coordinates 0.950-0.978). J. Gen. Virol. **62**:1-15.

Papavassiliou, A. G., K. W. Wilcox, and S. J. Silverstein. 1991. The interaction of ICP4 with cell/infected-cell factors and its state of phosphorylation modulate differential recognition of leader sequences in herpes simplex virus DNA. EMBO J. **10**:397-406.

O'Hare, P. and G. S. Hayward. 1985. Evidence for a direct role for both the 175,000- and 110,000-molecular-weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J. Virol. **53**:751-760.

Post, L. E. and B. Roizman. 1981. A generalized technique for deletion of specific genes in large genomes: gene 22 of herpes simplex virus 1 is not essential for growth. Cell **25**:227-232.

Rixon, F. J. and J. B. Clements. 1982. Detailed structural analysis of two spliced HSV-1 immediate-early mRNAs. Nucl. Acids Res. **10**:2241-2256.

Roizman, B., L. E. Carmichael, F. Deinhardt, G. de-The, A. J. Nahmias, W. Plowright, F. Rapp, P. Sheldrick, M. Takahashi, and K. Wolf. 1981. Herpesviridae: Definition, provisional nomenclature, and taxonomy. Intervirology **16**:201-217.

Roizman, B., Warren, J., C. A. Thuning, M. S. Fanshaw, B. Norrild, and B. Meignier. 1982. Application of molecular genetics to the design of live herpes simplex virus vaccines. Develp. Biol. Standard. **52**:287-304.

Roizman, B. 1990. Whither herpesviruses?, p 285-291. *In* C. Lopez et al. (ed.), Immunobiology and prophylaxis of human herpesvirus infections. Plenum Press, New York.

Ross, L.J.N., M. Sanderson, S. D. Scott, M. M. Binns, T. Doel, and B. Milne. 1989. Nucleotide sequence and characterization of the Marek's disease virus homologue of glycoprotein B of herpes simplex virus. J. Gen. Virol. **70**:1789-1804.

Ross, L. J. N. and M. M. Binns. 1991. Properties and evolutionary relationships of the Marek's disease virus homologues of protein kinase, glycoprotein D and glycoprotein I of herpes simplex virus. J. Gen. Virol. **72**:939-947.

Ross, L. J. N., M. M. Binns, and J. Pastorek. 1991. DNA sequence and organization of genes in a 5.5 kbp Eco RI fragment mapping in the unique short segment of Marek's disease virus (strain RB1B). J Gen. Virol. **72**:949-954.

Schat, K. A., A. Buckmaster,, and L.J.N. Ross. 1989. Partial transcription map of Marek's disease herpesvirus in lytically infected cells and lymphoblastoid cell lines. Int. J. Cancer **44**:101-109.

Scott, S. D., N.L.J. Ross, and M. M. Binns. 1989. Nucleotide and predicted amino acid sequences of the Marek's disease virus and turkey herpesvirus thymidine kinase genes; comparison with thymidine kinase genes of other herpesviruses. J. Gen. Virol. **70**:3055-3065.

Sears, A. E., I. W. Halliburton, B. Meignier, S. Silver, and B. Roizman. 1985. Herpes simplex virus 1 mutant deleted in the α22 gene: Growth and gene expression in permissive and restrictive cells and establishment of latency in mice. J. Virol. **55**:338-346.

Silva, R. F. and R. L. Witter. 1985. Genomic expansion of Marek's disease virus DNA is associated with serial in vitro passage. J. Virol. **54**:690-696.

Simek, S. and N. R. Rice. 1988. $p59^{v\text{-}rel}$. the transforming protein of reticuloendotheliosis virus, is complexed with at least four other proteins in transformed lymphoid cells. J. Virol. **62**:4730-4736.

Sithole, L., L. F. Lee, and L. F. Velicer. 1988. Synthesis and processing of the Marek's disease herpesvirus B antigen glycoprotein complex. J. Virol. **62**:4270-4279.

Solomon, J. J. 1975. Preparation of avian cell culture. Tissue Culture Methods **1**:7-11.

Vafai, A., R. S. Murray, M. Wellish, M. Devlin, and D. H. Gilden. 1988. Expression of varicella-zoster virus and herpes simplex virus in normal human trigeminal ganglia. Proc. Natl. Acad. Sci. USA **85**:2362-2366.

Watson, R. J., M. Sullivan, and G. F. Vande Woude. 1981. Structures of two spliced herpes simplex virus type 1 immediate-early mRNA's which map at the junctions of the unique and reiterated regions of the virus DNA S component. J. Virol. **37**:431-444.

Weber, P. C., M. Levine, and J. C. Glorioso. 1987. Rapid identification of nonessential genes of herpes simplex virus type 1 by Tn5 mutagenesis. Science **236**:576-579.

Whitton, J. L. and J. B. Clements. 1984. The junctions between the repetitive and the short unique sequences of the herpes simplex virus genome are determined by the polypeptide-coding regions of two spliced immediate-early mRNAs. J. Gen. Virol. **65**:451-466.

Wilcox, K. W., A. Kohn, E. Sklyanskaya, and B. Roizman. 1980. Herpes simplex virus phosphoproteins. I. Phosphate cycles on and off some viral polypeptides and can alter their affinity for DNA. J. Virol. **33**:167-182.

Wirth, U. V., B. Vogt, and M. Schwyzer. 1991. The three major immediate-early transcripts of bovine herpesvirus 1 arise from two divergent and spliced transcription units. J. Virol. **65**:195-205.

Zhang, G. and D. P. Leader. 1990. The structure of the pseudorabies virus genome at the end of the inverted repeat sequences proximal to the junction with the short unique region. J. Gen. Virol. **71**:2433-2441.

Chapter IV

Analysis of Marek's disease virus unique short region polypeptides with antisera to inducible, bacterially-expressed fusion proteins

ABSTRACT

Our recently completed nucleotide sequence of the Marek's disease virus (MDV) unique short region identified 7 alphaherpesvirus-related open reading frames (ORFs), 3 MDV-specific ORFs, and a novel fowlpox virus homolog (Brunovskis and Velicer, 1992a). To facilitate characterization of the polypeptides specified by these ORFs, monospecific, polyclonal antisera were generated from a panel of 16 different bacterially-expressed *trpE* fusion protein immunogens representing 9 of the 11 U_S region ORFs. The resulting antibodies were found to immunoprecipitate 6 of the 7 alphaherpesvirus-related U_S homologs from MDV-infected cell cultures. US2-specific antibodies precipitated a 30-kDa polypeptide; US10-specific antibodies, a 24-kDa phosphoprotein. Three different antisera were all found to precipitate a 47,49-kDa doublet corresponding to the MDV protein kinase-related product, US3; one of these antisera specifically reacted with a 68-kDa cellular protein as well. As in other alphaherpesvirus systems, antisera was found to coprecipitate 46- and 62-72-kDa glycoproteins homologous to HSV US7 (gI) and US8 (gE), respectively. Boiling of lysates in 1% SDS/2-mercaptoethanol failed to prevent the coprecipitation of gI and gE. This suggests that, like , MDV gI and gE share common epitopes in a similar fashion as their related varicella-zoster homologs. Expression of polypeptides encoded by US3, -7, -8, and 10 was inhibited by phosphonoacetic acid treatment; this suggests that they are late proteins, possibly associated with virion components. Antibodies reactive with the US6 (gD) epitopes of three different bacterially-expressed fusion proteins failed to precipitate gD from avian cell cultures. A consideration of the results obtained raises a number of interesting questions highlighting the potential importance of U_S region genes in determining a number of MDV's unusual biological properties.

INTRODUCTION

Marek's disease virus (MDV) is an avian herpesvirus that can cause T cell lymphomas as quickly as three weeks postinfection (for a recent review, see Calnek and Witter, 1991). Although numerous studies have addressed its complex pathogenesis, molecular biological studies have lagged behind those of other herpesvirus systems. The major difficulty of working with MDV is that it is strongly cell-associated and lacks a suitable cell culture system amenable to fully productive infections. Primary chick- or duck embryo fibroblasts are permissive for MDV replication. Such cell cultures are characterized by slowly progressing, semi-productive infections, which fail to result in the production of enveloped cell-free virus. These factors necessitate passage of infected cells onto uninfected cell monolayers in order to obtain sufficient quantities of material with which to work. This precludes straightforward plaque purification; consequently, isolation of mutants is much more difficult. Such conditions also preclude the establishment of one-step growth conditions for effective temporal gene regulation studies. Fully productive infections are restricted to the feather follicle epithelium (FFE); FFE-derived cell-free virus titers are generally limited to just 10^4 PFU/ml. Together, these difficulties help account for the characterization of only four genes and their gene products reported in the current literature. These correspond to the MDV homologs of the HSV glycoproteins gB (Chen and Velicer, 1992; Isfort et al., 1986b), gC (Binns and Ross, 1989; Isfort et al., 1986a; Coussens and Velicer, 1988), and two MDV-specific products, pp38 (Chen et al., 1992a; Cui et al., 1991) and *meq* (Jones et al., 1992).

Recent data demonstrating a closer phylogenetic relationship between MDV and alphaherpesviruses than between MDV and gammaherpesviruses

(Binns and Ross, 1989; Brunovskis and Velicer, 1992a; Buckmaster et al., 1988; Ross et al., 1989; Ross and Binns, 1991; Ross et al., 1991; Scott et al., 1989) has focused renewed attention on alphaherpesvirus systems as a source for clues to shed light on the many complexities and paradoxes associated with MDV. These related human and animal herpesvirus systems provide a wealth of useful information that can facilitate rapid progress in the MDV field. Complete nucleotide sequences are now available for three alphaherpesvirus members, herpes simplex virus (HSV; McGeoch et al., 1988), varicella zoster (VZV; Davison and Scott, 1986), and equine herpesvirus (EHV; Telford et al., 1992). Gene sequences are often of limited use in the absence of additional information relating to the characterization of their products. Many of the latter studies have been carried out with HSV; new information resulting from these studies provide a foundation for further studies of their related counterparts in other alphaherpesvirus systems.

We have recently determined the complete nucleotide sequence for the MDV U_S region (Brunovskis and Velicer, 1992a). Our analysis identified at least 11 open reading frames (ORFs) likely to code for proteins; of these, 7 represent homologs exclusively related to alphaherpesvirus S region genes. These include MDV counterparts of HSV US1 (ICP22), US2, US3 (protein kinase), US6, US7 and US8 (glycoproteins gD, gI and gE, respectively) and US10. Three additional ORFs were identified with no apparent relation to any sequences found among herpesviruses or present in the existing databases, while a fourth was found to be homologous to a fowlpox virus ORF.

Since the U_S region exhibits significant genetic diversity, pathogenic potential, and the presence of functions nonessential for growth in cell culture, further MDV studies involving U_S region genes are likely to shed light on the different pathogenic expressions associated with members of the

alphaherpesvirus subfamily (Brunovskis and Velicer, 1992a). Unlike HSV or VZV, the MDV system offers unique opportunities for studying the interaction of these genes in their natural host. The facilitation of such studies often requires suitable immunologic reagents. In this report we present a preliminary analysis of MDV U_S region polypeptides utilizing such reagents. We have employed inducible expression vectors (pATH series; Koerner et al., 1991) to create a series of *trpE*-MDV fusion protein immunogens. Sixteen different fusion proteins corresponding to nine of the eleven MDV U_S ORFs were expressed in *E. coli*. Our preliminary analysis succeeded in characterizing a group of polyclonal antibody preparations capable of precipitating all seven MDV U_S region alphaherpesvirus homologs.

MATERIALS AND METHODS

Cells and viruses. The preparation and cultivation of primary duck embryo fibroblast (DEF) cells was by established methods (Solomon, 1975). Pathogenic MDV GA strain-infected cells (passage level 6) and HVT vaccine virus strain FC-126-infected cells (passage level 13) frozen in liquid nitrogen were used to infect 85-90% confluent DEF cell monolayers at a 1:6-1:8 dilution in growth medium initially containing 4% calf serum. In some experiments, cells were treated with phosphonoacetic acid (PAA; 200 μg/ml; Lee et al., 1976) at 12 hr postinfection.

Antisera. Immune chicken sera (ICS) was pooled together from convalescent chickens naturally exposed to MDV. RαUS1 sera has been described (Brunovskis and Velicer, 1992b). Normal rabbit serum (NRS), normal chicken serum (NCS) from specific pathogen-free (SPF) chickens, and rabbit anti-chicken IgG were obtained from Sigma Chemical Co. Antisera generated from this study are described in the text.

Cloning of plasmid constructs for bacterial fusion protein expression. Sequence analysis of the MDV U_S region (Brunovskis and Velicer, 1992a) led to the identification of useful restriction sites (Figure 1) for cloning of in-frame fusions with the amino terminal 323-aa of *trpE*. MDV inserts were gel-purified (LMP agarose), blunt-ended, when necessary, and ligated into the multiple cloning site of the appropriate pATH expression vector (kindly provided by R. C. Schwartz); $CaCl_2$-competent *E. coli* RR1-transformed cells were mini-prep-screened to facilitate isolation of the desired recombinants.

Expression and purification of fusion protein immunogens. Induced expression and purification of *trpE*-fusion proteins was carried out according to a recently published procedure (Koerner et al., 1991).

Immunizations with *trpE* fusion proteins. 1.0-1.5 mg of 1x sample buffer-solubilized *trpE* fusion proteins were electrophoresed through 3-mm thick 7.5% SDS-PAGE gels (preparative) and stained with Coomasie Blue. Fusion proteins were washed in distilled water for 1 hr and homogenized in 1-2 volumes of Freund's complete- or incomplete adjuvant by serial passage through 18-, 20-, 22- and 25-gauge syringe needles. Primary injections (subcutaneous route) of female New Zealand white rabbits involved the use of 750 μg of fusion protein in Freund's complete adjuvant; subsequent injections employed 375 μg of fusion protein in Freund's incomplete adjuvant. The rabbits were boosted once a month; serum was collected 10-14 days following each boost.

Radiolabeling of proteins. Mock- and virus-infected DEF cells were labeled with 50 μCi/ml of [^{35}S]-methionine (specific activity, 1,000 μCi/mmol, ICN) at 48-72 hrs. post-infection for 2-4 hrs. as previously described (Isfort et al., 1986b). A similar approach was employed for [^{32}P]-orthophosphate labeling of DEF cells, with the following modifications. Phosphate-free Dulbecco modified Eagle medium was used for 3 hrs before- and during the 2 hr labeling period in which 250 μCi/ml $^{32}P_i$ (carrier-free; Amersham Corp.) was used per 60 mm plate.

Labeled bacterial lysates containing *TrpE*- and *trpE*-fusion proteins were grown similar to the large-scale harvests (Koerner et al., 1991) with the following modifications. Overnight cultures were inoculated from frozen stocks into media supplemented with each of the 20 aa at a concentration of 50 μg/ml. The following day, 0.5 ml was inoculated with 4.5 ml of the above media lacking methionine and tryptophan and incubated by vigorous shaking in a 50 ml tube at 37 C for 2 hr. The cells were then treated with 10 μg/ml indoleacrylic acid (IAA), and incubated for an additional hr prior to the addition of 250 μCi [^{35}S]-methionine. Following a 15 min pulse at 37 C, cells were pelleted at 1,000g and

lysed with 1 ml of 1x phospholysis buffer (PLB; Witte and Wirth, 1979) supplemented with protease inhibitors as previously described (Brunovskis and Velicer, 1992b). 50 μl of lysate was used for each immunoprecipitation.

Immunoprecipitation and SDS-PAGE analyses. Immunoprecipitation/SDS-PAGE analyses were carried out as previously described (Isfort et al., 1986b). The specificity of *trpE*-MDV-directed antisera was tested by preincubating 970 μl PBS with 10 μl of *trpE* fusion antisera and 20 μl 1x sample buffer-solubilized *trpE*-fusion proteins (or *trpE* alone) for 30 min-1 hr at 4 C. Labeled cell lysates were then added and processed as above. In some cases, lysates were adjusted to 1% SDS, 1% 2-mercaptoethanol (2-ME) and boiled for 5 min prior to the addition of antibodies (Simek and Rice, 1988). When using chicken antibodies, an equal volume of rabbit anti-chicken IgG was added for at least 1 hr prior to the Staph A precipitation step. Immunoprecipates were washed 2x with 1x PLB and electrophoresed on 10% SDS-PAGE gels, unless otherwise noted. High-stringency wash conditions involved sequential washes as follows; twice with RIPA buffer; once with high salt wash (2M NaCl, 10 mM Tris-HCl, pH 7.4, 1% NP40, 0.1% SDS); once with 1M $MgCl_2$; once with Tris-HCl, pH 7.4. ^3H-labeled protein markers (BRL) were included for estimation of molecular weights.

RESULTS

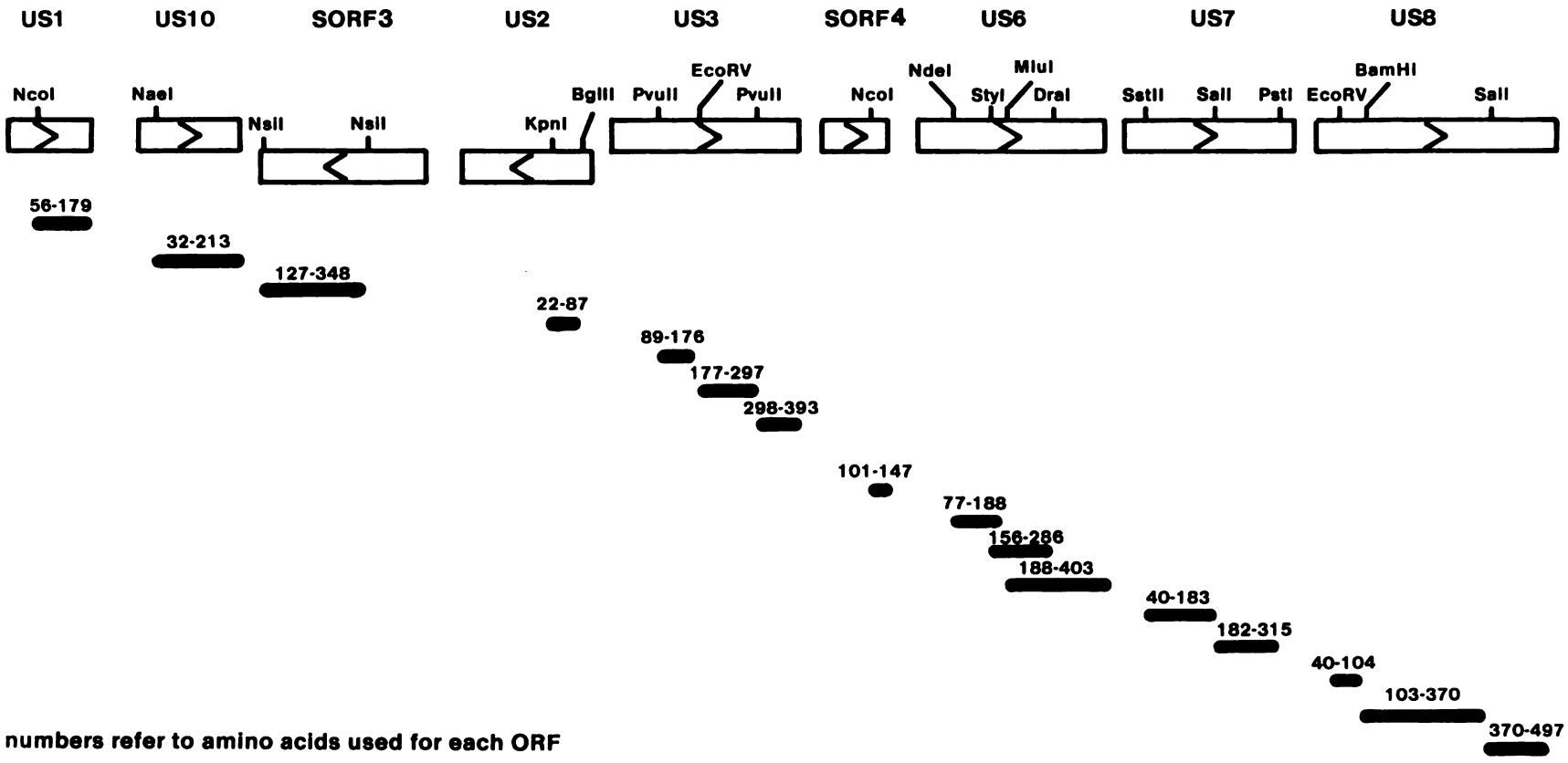
Nine of the eleven MDV U_S ORFs were targeted for antibody production (Figure 1). Not included were the two most recently identified ORFs, SORF1 and SORF2 (Brunovskis and Velicer, 1992a). The antisera generated was immunoreactive with all 7 of the alphaherpesvirus-related U_S region polypeptides. US1 polypeptide expression is described elsewhere (Brunovskis and Velicer, 1992a).

MDV US10. HSV US10 encodes a putative virion protein (McGeoch et al., 1988) containing a potential zinc-finger domain (Holden et al., 1992) that appears to be nonessential for replication in cell culture. Two rabbits were independantly immunized with *trpE*-US10. The antisera generated (RαUS10) was capable of immunoprecipitating a 24-kDa MDV-specific polypeptide from infected cells. The protein was detected with either [^{35}S]-methionine (Figure 2A, lanes 2 and 3) or [^{32}P]-orthophosphate (Figure 2C, lane 10) labelings at late times postinfection (72 hr) when significant cytopathic effects were observed. The immunoprecipitation appeared to be specific; preincubation of RαUS10 antibodies with *trpE*-US10 (but not *trpE* alone) blocked the precipitation (Figure 2B, lanes 5, 6). Lack of reactivity with HVT-infected cell lysates (data not shown) suggests that HVT lacks a US10 homolog. Nucleotide sequencing of HVT's S region should resolve this point. Expression of the 24-kDa polypeptide was sensitive to PAA treatment of infected cells (Figure 2D); PAA-sensitive and PAA-insensitive controls are outlined in Figure 4C, below. This result indicates that MDV US10 encodes a late protein, consistent with the expected properties of its HSV-1 counterpart, previously described as a virion protein (McGeoch et al., 1988).

FIGURE 1. Map of MDV regions fused to *trpE* in this study.

ORFs are according to (Brunovskis and Velicer, 1992a). Restriction sites used for ligating MDV coding sequences to *trpE* are displayed, as are their corresponding amino acid sequence numbers.

MAP OF MDV REGIONS FUSED TO TrpE IN THIS STUDY



numbers refer to amino acids used for each ORF

FIGURE 2. Immunoprecipitation/SDS-PAGE analysis of the polypeptide encoded by MDV US10.

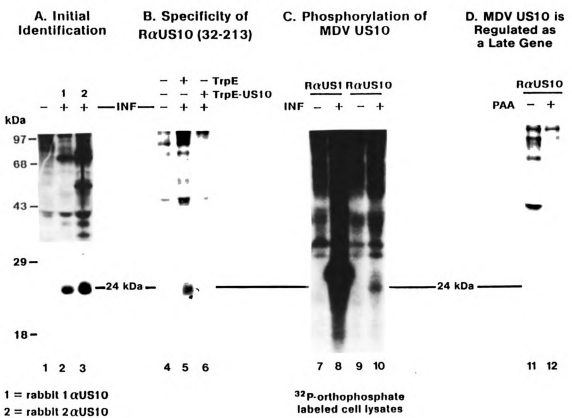
A) Identification of the MDV US10 polypeptide. Lysates from mock-infected cells were analyzed by immunoprecipitation with pooled sera from rabbits 1 and 2 immunized with *trpE*-US10 (lane 1); MDV-infected cell lysates (lanes 2,3) were analyzed with antiseras from rabbits 1 and 2, respectively; subsequent experiments employed RαUS10 from rabbit 1.

B) Specificity of RαUS10 antiserum. Lysates from mock (lane 1)- or MDV (lanes 2,3)-infected cells were analyzed by immunoprecipitation with RαUS10 (lane 1) or RαUS10 preincubated with either *trpE* (lane 2) or *trpE*-US10 (lane 3).

C) Phosphorylation of US10. Lysates from mock (lanes 1,3)- or MDV (lanes 2,4)-infected cells labeled with [^{32}P]-orthophosphate were analyzed by immunoprecipitation with RαUS1 (Brunovskis and Velicer, 1992b), lanes 1,2 or RαUS10, lanes 3,4.

D) MDV US10 is regulated as a late polypeptide. Infected cell lysates from untreated (lane 11) or PAA-treated (lane 12) cells were analyzed by immunoprecipitation with RαUS10. PAA controls are described in Figure 4C below.

IMMUNOPRECIPITATION/SDS-PAGE ANALYSIS OF THE POLYPEPTIDE ENCODED BY MDV US10



MDV US2. Nothing is known about the nature of US2 homologs other than the fact that they appear to be dispensable for replication in cell culture (Cantello et al., 1991; Weber et al., 1987). RαUS2 was found to specifically immunoprecipitate a 30-kDa MDV US2 homolog (Figure 3A, lane 2); an antibody-blocking experiment was similarly performed to demonstrate the specificity of RαUS2 (Figure 3B).

US3 (PK). MDV codes for a product (Brunovskis et al., 1992; Ross et al., 1991) which has a close relationship to the HSV US3-encoded protein kinase (PK) (McGeoch et al., 1985; McGeoch and Davison, 1986) and its PRV- (van Zijl et al., 1990; Zhang and Leader, 1990), VZV- (Davison and Scott, 1986; McGeoch and Davison, 1986), and EHV-1 (Colle et al., 1992) counterparts. These products exhibit significant homology to the serine-threonine PK superfamily (Hanks et al., 1988; Leader and Purves, 1988). The MDV PK-specific antisera, RαPK1, RαPK2, and RαPK3 were directed against three different regions of the polypeptide as depicted in Figure 1. Under standard wash conditions (2-1xPLB washes) these antisera were found to immunoprecipitate several polypeptides, including a 47,49-kDa doublet present in infected cells (Figure 4A, lanes 2,4,6) and a 68-kDa polypeptide present in uninfected cells (Figure 4A, lane 1). Additional polypeptides, possibly less related serine-threonine PKs, were precipitated as well. To clarify the nature of the MDV-encoded PK product(s), higher stringency wash conditions were employed (Figure 4B; see Materials & Methods). As a further test for specificity, an antibody-blocking experiment was conducted as before. US3 antisera 177-297 and 298-393 were found to specifically precipitate a 47,49-kDa doublet in MDV-infected cells (Figure 4B, lanes 6 and 10). With these wash conditions antiserum 89-176 was found to primarily precipitate a 68-kDa cellular polypeptide; once again, the reaction appeared specific, since *trpE*-US3/89-176 blocked this precipitation, while *trpE*

FIGURE 3. Immunoprecipitation/SDS-PAGE analysis of the polypeptide encoded by MDV US2.

A) Identification of the MDV US2 polypeptide. Lysates from mock (lane 1)- or MDV (lanes 2)-infected cells were analyzed by immunoprecipitation with RαUS2.

B) Specificity of RαUS2 antiserum. Lysates from mock (lane 1)- or MDV (lanes 2,3)-infected cells were analyzed by immunoprecipitation with RαUS2 (lane 1) or RαUS2 preincubated with either *trpE* (lane 2) or *trpE*-US2 (lane 3).

IMMUNOPRECIPITATION/SDS-PAGE ANALYSIS OF THE POLYPEPTIDE ENCODED BY MDV US2

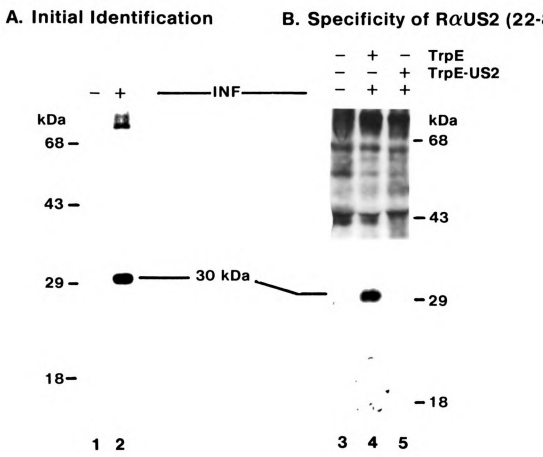


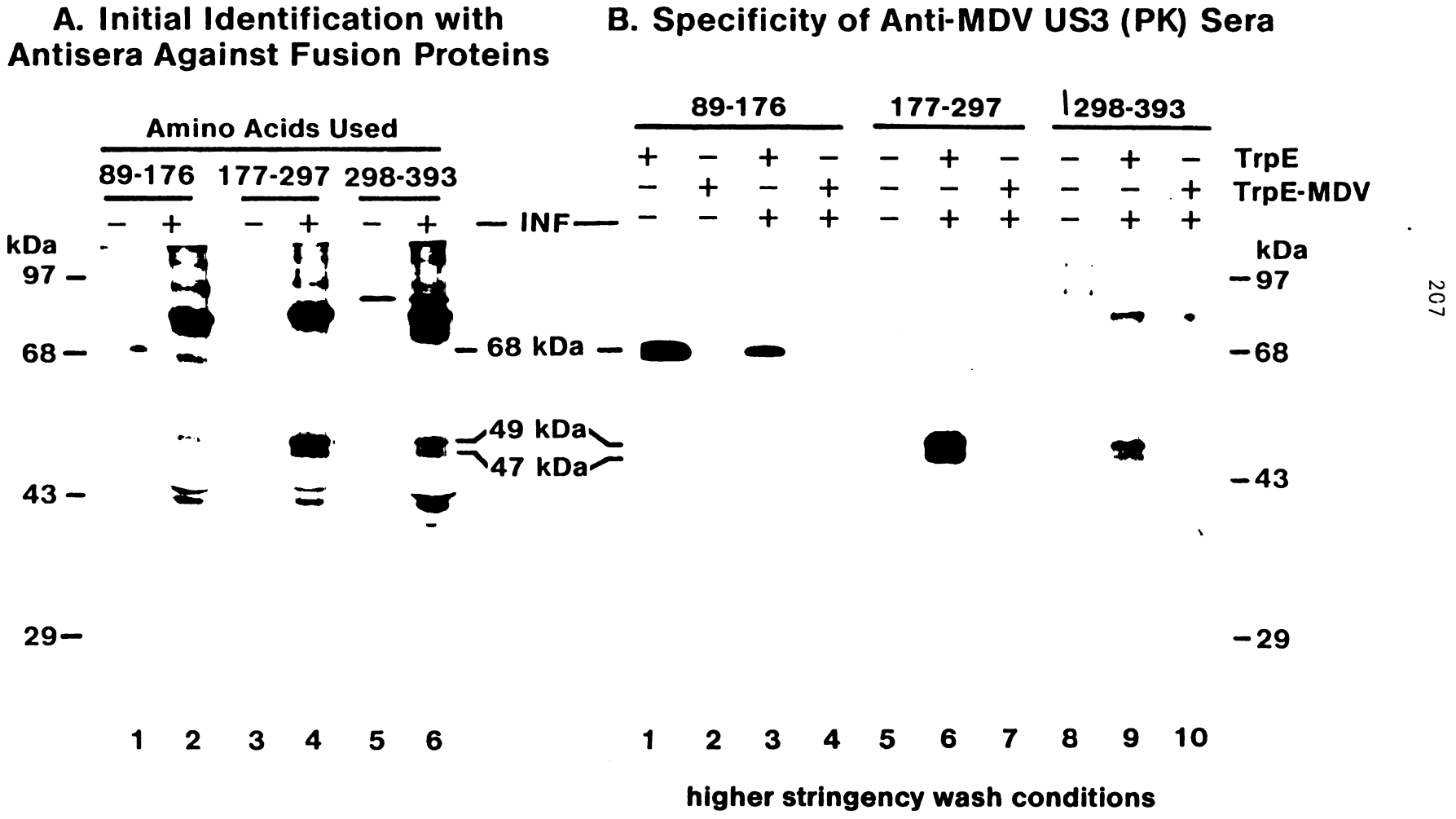
FIGURE 4. Immunoprecipitation/SDS-PAGE analysis of the polypeptide encoded by MDV US3 (PK).

A) Initial identification. Lysates from mock (lanes 1,3,5)- and MDV (lanes 2,4,6)-infected cells were analyzed by immunoprecipitation with different PK antisera as indicated.

B) Specificity of RαPK antisera. Lysates from mock (lanes 1,2,5,8)- and MDV (lanes 3,4,6,7,9-11)-infected cells were analyzed by immunoprecipitation with RαPK sera (lanes 5,8) or with RαPK sera preincubated with either *trpE* (lanes 1,3,6,9) or the respective *trpE* fusion protein from which they derived (lanes 2,4,7,10), as indicated. Higher stringency wash conditions were employed (see Materials and Methods).

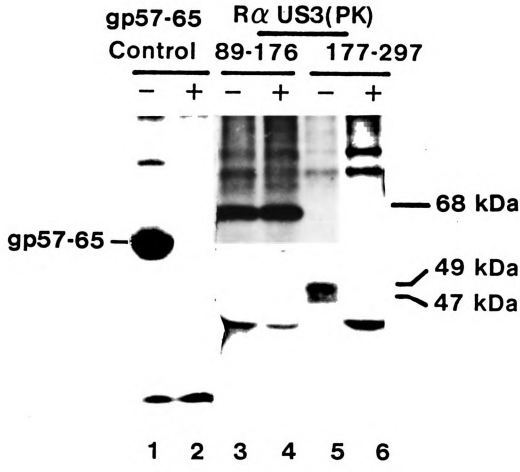
C) Temporal regulation of MDV PK. Infected cell lysates from untreated (lanes 1,3,5) or PAA-treated (lanes 2,4,6) cells were analyzed by immunoprecipitation with RαPK seras, as indicated (lanes 1-4). For a PAA-sensitive late gene control, ICS was used to precipitate MDV gp57-65/gC (lanes 5,6). RαUS3/89-176 provided a useful internal control, since it immunoprecipitates a cellular protein (see Figure 3B), whose expression is insensitive to PAA.

IMMUNOPRECIPITATION/SDS-PAGE ANALYSIS OF THE POLYPEPTIDE ENCODED BY MDV US3



MDV US3 ANALYSIS (CONT.)

C. MDV US3 (PK) is Regulated as a Late Gene



alone did not (Figure 4B, compare lanes 1 and 2). This same antiserum could also detect the 47,49-kDa doublet from infected cells using regular wash conditions (Figure 4A, lane 2) or high stringency wash conditions (Figure 4C, lane 3); detection in the latter required longer exposure times.

To see whether expression of the MDV PK doublet was dependent on viral replication, immunoprecipitations were carried out using cells treated with or without PAA. In the presence of PAA, MDV PK expression appeared to be completely shut down (Figure 4C, compare lane 3 with 4 and 5 with 6). Expression of the gp57-65 late gene control was similarly affected (lanes 5,6). These results were not attributed to a general inhibitory effect, since PAA had no effect on the expression of the 68-kDa cellular protein (lanes 1 and 2). These results indicate that MDV PK is regulated as a late (gamma) gene.

MDV US7 (gI) and US8 (gE). These two glycoproteins are grouped together to reflect their close association with one another. Both glycoproteins have been found to coprecipitate as a complex in HSV infected cells (Johnson and Feenstra, 1987; Johnson et al., 1988), PRV (Zuckermann et al., 1988), VZV (Vafai et al., 1988b; Vafai et al., 1989); in the case of VZV, these two glycoproteins appear to share a common epitope (Vafai et al., 1988b; Vafai et al., 1989). Immunoprecipitation analysis of infected cell lysates with antibodies against the amino- and carboxy regions of MDV gI identified MDV-specific glycoproteins 45- and 62-72-kDa in size (Figure 5, lanes 3,6). An antibody-blocking assay demonstrated the specificity of this precipitation. Subsequent analyses employing gE-directed antibodies resulted in the precipitation of the same 62-72-kDa polypeptides first identified with RαUS7, in this case unassociated with gI (Chen et al., 1992b). That these polypeptides represent the same ones precipitated with RαUS7 antibodies is evidenced by failure to precipitate gE with RαUS8 following preclearance of lysates with RαUS7 (data

FIGURE 5. Immunoprecipitation/SDS-PAGE analysis of the glycoproteins encoded by MDV US7 (gI) and US8 (gE).

A) Specificity of RαUS7 and coprecipitation of MDV gI and gE. Lysates from mock (lanes 1,4)- and MDV (lanes 2,3,5,6)-infected cells were analyzed by immunoprecipitation with the indicated RαUS7 sera (lanes 1-6) preincubated with either *trpE* (lanes 2,5) or with the respective *trpE* fusion protein from which they were derived (lanes 3,6). High stringency wash conditions were employed (see Materials and Methods).

B) Coprecipitation after boiling with 1% SDS/2-ME. Infected cell lysates were untreated (lane 7) or boiled for 5 min in 1% SDS, 1% 2-ME prior to immunoprecipitation analysis with RαUS7/182-315.

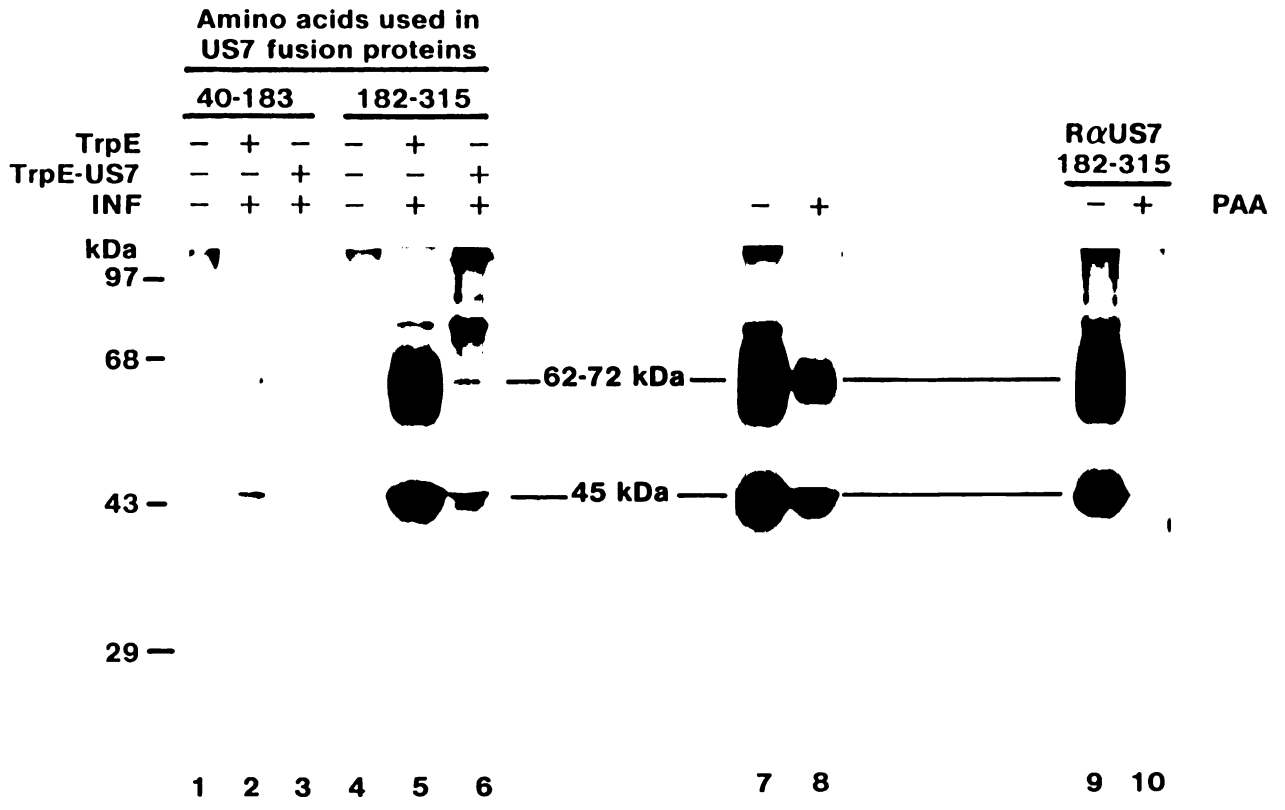
C) MDV gI and gE are regulated as late genes. Infected cell lysates from untreated (lane 9) or PAA-treated (lane 10) cells were subjected to immunoprecipitation analysis with RαUS7/182-315. PAA controls are described in Figure 4C above.

IMMUNOPRECIPITATION/SDS-PAGE ANALYSIS OF THE GLYCOPROTEINS ENCODED BY MDV US7 (gI) and US8 (gE)

A. Specificity of RαUS7 and coprecipitation of MDV gI and gE

B. Coprecipitation after boiling with 1% SDS/2-ME

C. MDV gI and gE are regulated as late genes



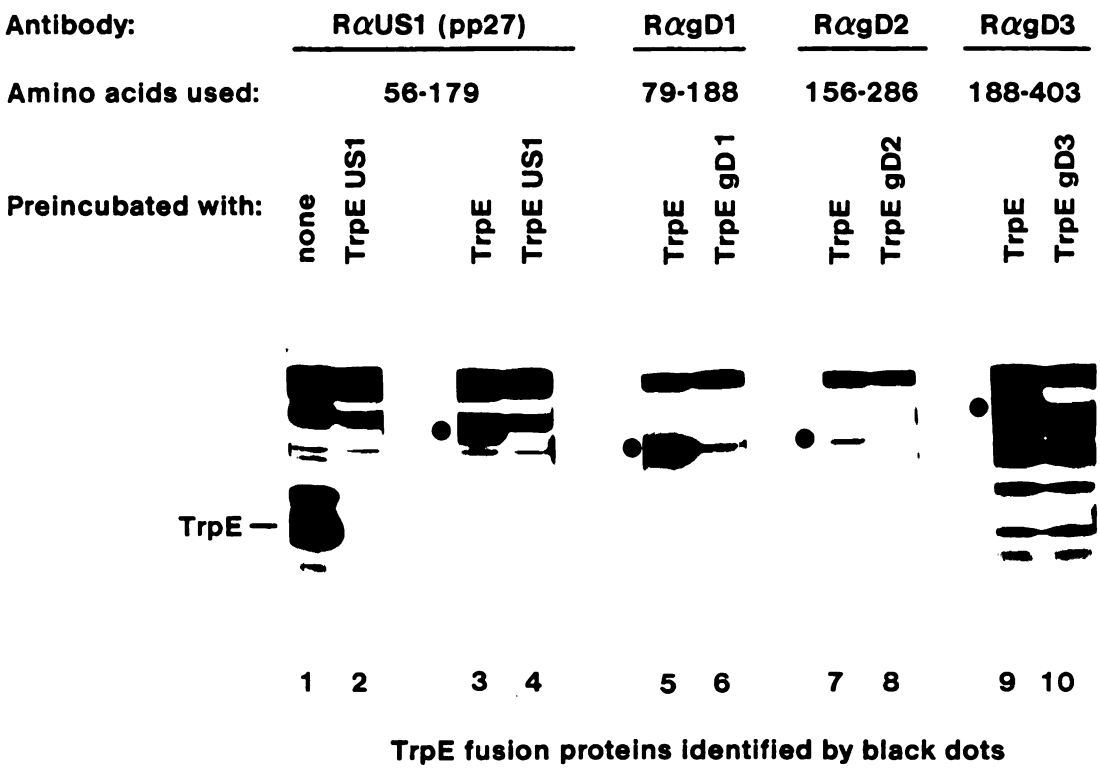
not shown). Further evidence suggests that RαUS7 antibodies recognize common epitopes in gI and gE. This was based on the observation that gI and gE both maintained their coprecipitability after boiling of lysates in the presence of 1% SDS and 1% 2-mercaptoethanol (Figure 5B). Expression was further found to be sensitive to PAA treatment. This indicates that MDV gI and gE are regulated as late genes.

MDV US8 (gD). As with MDV PK, rabbits were immunized with three different fusion proteins designed to react with distinct regions of MDV gD (Figure 1). Despite numerous attempts and a number of different labeling regimens, specific, immunoreactive gD products could not be detected in infected DEF cells. To examine whether this negative result reflected a lack of gD immunoreactivity, a number of different lysates from *trpE-* or the *trpE* gD-transformed *E. coli* radiolabeled with [^{35}S]-methionine were subjected to immunoprecipitation analysis with gD-directed antibodies preincubated with SDS-PAGE loading buffer alone, *trpE* or the different *trpE*-gDs as indicated (Figure 6). Since fusion protein antisera react with *trpE* epitopes (Figure 6, lane 1) it was important to set up these preincubations in order to ascertain that the reactivity was not only directed against *trpE* epitopes in the fusion protein but against gD epitopes as well. Validation of this assay required a means to specifically block the *trpE*-reactive antibodies. As shown in Figure 6, lane 2, preincubation of RαUS1 antibodies with *trpE* protein completely blocked the ability of anti-*trpE* antibodies present in the preparation to precipitate *trpE*. As a control for this assay, a known trpE-MDV-reactive antibody (RαUS1) was employed, which was previously shown to precipitate a 27,24-kDa ICP22-related polypeptide (encoded by US1) from MDV-infected cells (Brunovskis, 1992b). This assay was based on the premise that MDV-specific antibodies would maintain their ability to bind and immunoprecipitate the fusion proteins following

FIGURE 6. Immune-reactivity of *trpE*-gD-directed antibodies.

E. coli expressing *trpE* (lanes 1,2), *trpE*-US1 (lanes 3,4), *trpE*-gD1/79-188 (lanes 5,6), trpE-gD2/156-286 (lanes 7,8) or trpE-gD3/188-403 (lanes 9,10) were labeled with [^{35}S]-methionine as described in Materials and Methods. The labeled cell lysates were subjected to immunoprecipitation analysis with various antisera (as indicated) preincubated with either: (i) 1x sample buffer (lanes 1); (ii) *trpE* (lanes 2,3,5,7,9); (iii) *trpE*-US1 (lane 4); (iv) *trpE*-gD1/79-188 (lane 6); (v) *trpE*-gD2/156-286 (lane 8); or (vi) *trpE*-gD3/188-403 (lane 10).

IMMUNE-REACTIVITY OF TrpE-gD-DIRECTED ANTIBODIES



TrpE fusion proteins identified by black dots

blockage of the *trpE*-directed antibodies with *trpE* protein; the presence of MDV-specific antibodies would be verified by an inability to precipitate the identical fusion protein under similar conditions in which *both trpE*- and MDV-directed antibodies are blocked following preincubation with the *trpE*-fusion protein. As shown in Figure 6, lane 3, preincubation of RαUS1 control serum with *trpE* was not able to block the ability of MDV US1-directed antibodies from immunoprecipitating the fusion protein. However, the precipitation by US1-directed antibodies was completely lost following preincubation with *trpE*-US1 (lane 4). Similarly, while preincubations with *trpE* did not prevent the immunoprecipitation of the three *trpE*-gD fusion proteins (lanes 5,7,9), preincubations with *trpE*-fusion proteins did prevent these precipitations (lanes 6,8,10). These results demonstrated that the failure detect gD in infected DEF cells was not due to a lack of immunoreactivity.

Failure to detect gD polypeptides with antibodies directed against bacterially-expressed fusion proteins might be attributable to bacterially-derived epitope specificities masked by secondary structures present in the native, eukaryotically-expressed polypeptide. However, boiling of lysates prior to the addition of antibodies (to disrupt the secondary structure) did not facilitate detection of gD (data not shown). While it is conceivable that gD detection may require antibodies recognizing certain discontinuous epitopes not present in the bacterially-expressed fusion proteins, such an argument appears questionable in view of the overall success of the *trpE* system for detecting other MDV proteins (Table 1). Further Northern blot analysis has confirmed the apparent lack of gD expression. A radioactively labeled probe corresponding to the insert used in the *trpE*-gD1 construction (Figure 1) failed to detect any mRNAs in MDV-infected cells (data not shown). However, the same mRNA preparation did, however, hybridize to a probe corresponding to the insert used in the *trpE*-gD2

construction (Figure 1). These results suggest transcriptional initiation from a site within the gD ORF, possibly specifying transcripts for downstream ORFs, such as US7 (gI) and US8 (gE).

DISCUSSION

Our current study illustrates the practicality of employing the *trpE* expression system to generate polyclonal, monospecific antibodies to facilitate identification and characterization of new proteins predicted from sequence analysis (e.g. Brunovskis and Velicer, 1992a). Table 1 summarizes the polypeptides identified with the antibodies generated herein. The antibodies generated allowed for the successful immunoprecipitation of six of the seven MDV alphaherpesvirus-related U_S region products from infected DEF cell cultures. Antisera for two of the MDV-specific ORFs (SORF2 and -3) require further analysis; the two most recently identified U_S region genes (SORF1 and -2) still await the construction of suitable fusion protein-expressing plasmid clones. Analysis of US1 (ICP22), US7 (gI) and US8 (gE) homologs is described in greater detail elsewhere (Brunovskis and Velicer, 1992b; Chen et al., 1992b). The antibodies described in this report allow for a comparative analysis between MDV U_S region polypeptides and their alphaherpesvirus-related counterparts.

US10. The antiserum against the 24-kDa MDV US10 homolog is the first monospecific antibody preparation for any US10 homolog to date. HSV US10 has been characterized as a 33-kDa polypeptide identified by hybrid selection and cell-free translation (Lee et al., 1982; Rixon et al., 1984) and has been described as a virion protein (McGeoch, 1988), that is nonessential for replication in cell culture (Brown and Harland, 1987; Longnecker and Roizman, 1986; Umene, 1986). Immunoprecipitation analysis of HVT-infected cell lysates with antibodies to MDV US10 were negative. Because of the close immunologic- and phylogenetic relationship between MDV and HVT, failure to precipitate an HVT product suggests that HVT lacks a US10 homolog. This would not be surprising since HVT's U_S region is nearly 4 kb shorter than

Table 1. POLYPEPTIDES AND GLYCOPROTEINS IDENTIFIED IN THIS STUDY

Name	Length (aa)	Amino acids in fusion protein	Predicted molecular size (kDa)[a]	Molecular size(s) (kDa)
SORF1	89	NOT DONE	10.1	NOT DONE
SORF2	179	NOT DONE	20.1	NOT DONE
US1	179	56-179	20.4	21, 24[b], 27[b]
US10	213	32-213	23.6	24[b]
SORF3	351	127-348	40.6	c
US2	270	22-87	29.7	30
US3 (PK)	402	89-176	44.7	47, 49, 68[d]
		177-297		47, 49
		298-393		47, 49
SORF4	147	101-147	16.8	c
US6 (gD)	403	77-188	42.6[f]	e
		156-286		e
		188-403		e
US7 (gI)	355	40-104	38.3[f]	35[g], 45[h]
		103-370		35[g], 45[h]
US8 (gE)	497	40-104	53.7[f]	e, e
		103-320		45[g], 62-72[h]
		320-497		45[g], 62-72[h]

[a]In absense of post-translational modifications.
[b]Phosphorylated.
[c]Further analysis needed to verify identification.
[d]Cellular protein.
[e]No polypeptide found in MDV-infected primary DEF cell cultures.
[f]Based on sequences that follow the predicted signal peptide cleavage sites.
[g]With tunicamycin treatment.
[h]Without tunicamycin treatment

MDV's (Brunovskis and Velicer, 1992a; M. Wild, pers. comm.), yet it contains a number of the same homologs as MDV, including those specifying US7 (gI) and US8 (gE) (Chen et al., 1992b). Lack of a US10 homolog could contribute to the apathogenic nature of HVT.

Based on their analysis of a region highly conserved among alphaherpesvirus US10 homologs, Holden et al. (1992) have suggested that they share a potential zinc-finger domain, possibly implicating a role in gene regulation. Products involved in gene regulation are often regulated by phosphorylation. Phosphorylation of MDV US10 (Figure 3C) is consistent with a possible role in gene regulation.

US2. Identification of the 30-kDa MDV US2 homolog follows the recent identification of the only other US2-related polypeptide studied thus far (van Zijl, 1990). The absence of published reports concerning an HSV counterpart underscores the limited information regarding this product, which appears to be nonessential for replication of HSV-1 (Weber et al., 1987) and MDV (Cantello et al., 1991) in vitro. Unlike HSV-1 U_S mutants in mice (Meignier et al., 1988), PRV US2 mutants fail to exhibit reduced virulence in pigs (Kimman et al., 1992). All of the US2 homologs contain glycine at their N-terminus, indicative of a possible target for myristylation (Towler et al., 1987). Together with a notably hydrophobic N-terminal region, these properties might be expected to play a role in mediating transient membrane interactions (Schultz and Oroszlan, 1984).

US3 (PK). MDV PK was found to be expressed as a 47/49-kDa doublet, analogous to the larger 68/69-kDa doublet encoded by HSV-1 US3 (Zhang and Leader, 1990). Loss of PK expression following PAA treatment indicates that MDV PK specifies a late gene product, possibly representing a virion structural component. US3 PK-related products of HSV-1 and PRV have recently been identified in purified virions (Zhang and Leader, 1990). Such virions are known

to contain at least two potential PK substrates. One of these is encoded by the apparently essential (Purves et al., 1991) UL34 (McGeoch et al., 1988) gene product. Mutagenesis of the consensus US3 PK phosphorylation site (Leader et al., 1991) in UL34 was found to alter its response to US3 PK-mediated phosphorylation and resulted in severely impaired growth properties (Purves et al., 1991). Although HSV-1 and PRV PK mutants are known to grow in cell culture, impaired growth has been cited in both cases (de Wind et al., 1990; Purves et al., 1991). PRV and HSV-1 PK mutants also exhibit a significantly decreased virulence in pigs (Kimman et al., 1991) and mice (Meignier et al., 1988), respectively. The PRV PK mutant was further found to display an altered morphogenesis, possibly contributing to its lower level of replication in vitro (de Wind et al., 1990) and in vivo (Kimman et al., 1992). The use of immunological reagents specific for MDV PK should facilitate similar studies in the MDV system.

One of the biggest surprises of this study was the immunoprecipitation of a 68-kDa polypeptide from uninfected DEF cells using one of the three antisera directed against MDV PK (aa 89-176). The binding of this cellular product appeared to be specific, since its precipitation was blocked following preincubation of that antisera with *trpE*-PK, in contrast to *trpE* alone. The US3 PK-related family of alphaherpesvirus proteins appears to define a distinct subfamily within the serine-threonine protein kinase superfamily. It is thought that related cellular counterparts exist that are yet to be identified (Hanks et al., 1988). Further work will be necessary to ascertain whether the 68-kDa polypeptide represents such a product.

US7 (gI) and US8 (gE). The preliminary findings reported herein describe two glycoproteins (45- and 62-72-kDa) coprecipitated under conditions expected to denature and disassociate protein-protein complexes. Further work

reported elsewhere (Chen et al., 1992b) has established their glycoprotein identities. Both were labeled in the presence of glucosamine, and sensitive to treatment with the N-linked glycosylation inhibitor, tunicamycin. gI/gE complexes of HSV-1 and VZV have been found to act as Fc receptors (FcR) that can utilize an antibody bipolar bridging mechanism (Frank and Friedman, 1989) to protect HSV-infected cells from antibody-dependent cellular cytotoxicity (Dubin et al., 1991). MDV (Chen et al., 1992b) and PRV (Zuckermann et al., 1988) studies have thus far failed to associate FcR activity with gI/gE. However, both of the PRV products are known to specify important virulence determinants involved in dissemination of virus (Card et al., 1992; Kimman et al., 1992; Pol et al., 1991). Part of this is likely attributable to the role of gE in cell-cell spread (Zsak et al., 1992). Such findings are of significance to MDV studies, considering MDV relies on cell-cell spread in order to promote its dissemination in vivo. Similar to the case with PRV, (Quint et al., 1987), insertional inactivation of MDV's gE gene may represent a useful approach for developing new avirulent vaccine strains. However, considering MDV's reliance on cell-cell spread, such an approach may not allow for the appropriate level of replication required for inducing sufficient immune responses.

US6 (gD): Dispensable for MDV infections? Glycoprotein D (US6) is considered the only HSV-1 U_S region polypeptide essential for replication in cell culture (Roizman and Sears, 1990). This is a reflection of its obligate role in virus-cell penetration. Although PRV's gD homolog (gp50) is essential for penetration, unlike HSV, its production is not required for cell-cell spread (Peeters, 1992; Rauh, 1991). VZV, which is strongly cell-associated, lacks such a homolog altogether. Failure to detect MDV gD expression may have important implications related to the inability to facilitate fully productive infections in vitro and in vivo.

Like papillomaviruses, which are dependent on highly differentiated epithelial cells for expression of structural proteins and formation of virions, the production of fully-enveloped MDV virions is restricted to highly differentiated feather follicle epithelial (FFE) cells (Nazerian and Witter, 1970). Although it has been suggested that the keratinized environment of FFE cells may protect enveloped virus from lysosomal activity responsible for the destruction of newly-formed virions in other cells, such an explanation appears unlikely to account for failure to produce cell-free MDV in chick embryo fibroblast (CEF) cultures, since these cells support the cell-free growth of HSV and PRV.

The cell-cell spread observed in other tissues reflects a potentially effective strategy for immunoevasion by decreasing vulnerability to both humoral and cell-mediated effector mechanisms (Ahmed and Stevens, 1990). This type of dissemination occurs under conditions that appear to restrict full expression of the structural proteins necessary for envelopment and/or release. Reduced expression of viral glycoproteins has been described as an effective strategy for achieving persistent, non-productive infections with paramyxoviruses, retroviruses, retroviruses, rhabdoviruses and arenaviruses (Ahmed & Stevens, 1990). Abortive HSV infections of dog kidney cells are characterized by a 10-fold lower level of virus-induced surface antigens (Spring et al., 1968), while mutant BHK cells defective in enzymes of the Golgi system have been shown to exhibit a decrease in infectious HSV-1 yields, presumably due to a reduced ability to synthesize glycoproteins (Serafini-Cessi et al., 1983).

In vitro systems that promote fully productive MDV infections are not yet available. MDV studies generally rely on CEF or DEF culture sytems. Infection of these cells is characterized by slowly progressing, cell-associated, semi-productive infections. Over the last 20 years, studies to identify immunogenic surface antigens in the MDV system have relied on semi-productive cell culture

systems such as these. Although convalescent chicken sera may be expected to react with many, if not all, MDV-encoded surface antigens, this antisera would only allow for their detection to the extent that they are expressed in vitro. Conversely, rabbits or mice immunized with cell-culture-derived immunogens would only generate antibodies that reflect the potentially limited in vitro expression. Published reports cite just two MDV-encoded glycoproteins characterized as of yet. These correspond to the MDV homologs of HSV gB and gC. However, MDV codes for at least 3 additional glycoprotein genes (homologous to gD, gI and gE; Brunovskis and Velicer, 1992a; Ross et al., 1991); another has been reported for HVT (gH homolog; Buckmaster et al., 1988). Considering the abundant expression of gD in other alphaherpesvirus systems, it is surprising that the MDV glycoprotein counterpart has thus far escaped identification or detection. Seeking out such a polypeptide with the use of three different *trpE*-gD-directed antisera immunoreactive with their corresponding bacterially-expressed products, we could not identify it either (Figure 6). Furthermore, using a probe specific for the 5'-most coding sequences of gD, we failed to detect any transcripts (data not shown).

The apparent lack of gD expression suggests a potentially obligate role for gD in promoting fully productive infections in FFE cells. However, recent immunofluorescence studies employing *trpE*-gD directed antisera described in this report fail to support this view (R. Witter, pers. comm.). While such negative results are likely to raise questions about the validity of the reagents used, other lines of evidence question the need for gD. Recently, stable lacZ-MDV gD insertion mutants have been found to grow in vitro; moreover, these mutants could be rescued from chicken lymphocytes following cocultivation with uninfected fibroblasts (R. Morgan, pers. comm.).

Failure to express gD could benefit MDV in a number of ways. First, this would result in one less target for the immune system. Secondly, this would appear to promote a less lytic, less productive infection. This is important if MDV is to transform lymphocytes. More importantly, from MDV's point of view, lack of gD expression might actually *facilitate* horizontal transmission. The latter is mediated by the maturation and release of virus from FFE cells, usually by an air-borne route involving poultry house dust and chicken dander. Although the resulting virions are fully infectious in a cell-free form, release of these virions from FFE cells has never been observed. In contrast to cell-free virus from poultry dust sediments, which loses its infectivity in less than two weeks, intact poultry dust carrying desquamated MDV-containing FFE cells retains its infectivity for at least 205 days at ambient room temperatures (and much longer at lower temperatures) (Carozza et al., 1973). Based on these factors a compelling case can be made for *cell-associated* spread of MDV in horizontal transmission. By remaining cell-associated, albeit in a fully enveloped state, MDV appears to have a way of prolonging its period of infectivity.

Lack of gD expression may indeed account for MDV's cell-associatedness, based on evidence that HSV gD is involved in virus-cell release. HSV-1 gD has been reported to contain a domain which restricts fusion of the envelope with cytoplasmic membranes (Campadelli-Fiume et al., 1990). Alteration of this domain promotes cytoplasmic deenvelopment of virus upon egress, thereby resulting in the accumulation of unenveloped capsids alone or juxtaposed to cytoplasmic membranes (Campadelli-Fiume et al., 1991). Fibroblast cell cultures infected with MDV also accumulate unenveloped capsids (Nazerian et al., 1968), possibly reflecting an inability to prevent deenvelopment. Further MDV studies are likely to answer fundamental

questions about gD, that are of significance not only to the MDV system, but to other alphaherpesvirus systems as well.

REFERENCES

Ahmed, R. and J. G. Stevens. 1990. Viral persistence, p. 241-246. *In* B. Fields (ed.), Fields virology, vol. 1. Raven Press, New York.

Binns, M. M., and N.L.J. Ross. 1989. Nucleotide sequence of the Marek's disease virus (MDV) RB-1B A antigen gene and the identification of the MDV A antigen as the herpes simplex virus-1 glycoprotein C homologue. Virus Res. 12:371-382.

Brown, S. M. and J. Harland. 1987. Three mutants of herpes simplex virus type 2: One lacking the genes US10, US11 and US12 and two in which R_S has been extended by 6 kb to 0.91 map units with loss of U_S sequences between 0.94 and the U_S/TR_S junction. J. Gen Virol. 68:1-18.

Brunovskis, P. and L. F. Velicer. 1992a. Structural characterization of the Marek's disease virus (MDV) unique short region: Presence of alphaherpesvirus-homologous, fowlpox virus-homologous and MDV-specific genes. Submitted for publication.

Brunovskis, P. and L. F. Velicer. 1992b. Marek's disease virus expresses an unusual 27-kDa late class cytoplasmic phosphoprotein homologous to the 68-kDa herpes simplex virus 1 immediate-early nuclear phosphoprotein, ICP22. Manuscript in preparation.

Buckmaster, A. E., S. D. Scott, M. J. Sanderson, M.E.G. Boursnell, N.L.J. Ross, and M. M. Binns. 1988. Gene sequence and mapping data from Marek's disease virus and herpesvirus of turkeys: Implication for herpesvirus classification. J. Gen. Virol. 69:2033-2042.

Calnek, B. W., H. K. Adldinger, and D. E. Kahn. 1970. Feather follicle epithelium: a source of enveloped and infectious cell-free herpesvirus from Marek's disease. Avian Dis. 14:219-233.

Calnek, B. W. and R. L. Witter. 1991. Marek's disease, p. 342-385. *In* B. W. Calnek (ed.), Diseases of poultry, Iowa State University Press.

Campadelli-Fiume, G., S. Qi, E. Avitabile, L. Foa-Tomasi, R. Brandimarti, and B. Roizman. 1990. Glycoprotein D of herpes simplex virus encodes a domain which precludes penetration of cells expressing the glycoprotein by superinfecting herpes simplex virus. J. Virol. 64:6070-6079.

Campadelli-Fiume, G., F. Farabegoli, S. Di Gaeta, and B. Roizman. 1991. Origin of unenveloped capsids in the cytoplasm of cells infected with herpes simplex virus 1. J. Virol. 65:1589-1595.

Cantello, J. L., A. S. Anderson, A. Francesconi, and R. W. Morgan. 1991. Isolation of a Marek's disease virus (MDV) recombinant containing the lacZ gene of Escherichia coli stably inserted within the MDV US2 gene. J. Virol. 65:1584-1588.

Card, J. P., M. E. Whealy, A. K. Robbins, and L. W. Enquist. 1992. Pseudorabies virus envelope glycoprotein gI influences both neurotropism and virulence during infection of the rat visual system. J. Virol. 66:3032-3041.

Carozza, J. H., T. N. Fredrickson, R. P. Prince, and R. E. Luginbuhl. 1973. Role of desquamated epithelial cells in transmission of Marek's disease. Avian Dis. 17:767--781.

Chen, X., P.J.A. Sondermeijer, and L. F. Velicer. 1992a. Identification of a unique Marek's disease virus gene which encodes a 38-kilodalton phosphoprotein and is expressed in both lytically infected cells and latently infected lymphoblastoid tumor cells. J. Virol. 66:85-94.

Chen, X. and L. F. Velicer. 1992. Expression of the Marek's disease virus homolog of herpes simplex virus glycoprotein B in Escherichia coli and its identification as B antigen. J. Virol. **66**:4390-4398.

Chen, X. C., P. Brunovskis, and L. F. Velicer. 1992b. Identification and characterization of the Marek's disease virus homologs of the herpes simplex virus glycoproteins I and E. Submitted for publication.

Colle III, C., C. C. Flowers, and D. O'Callaghan. 1992. Open reading frames encoding a protein kinase, homolog of glycoprotein gX of pseudorabies virus, and a novel glycoprotein map within the unique short segment of equine herpesvirus type 1. Virology **188**:545-557.

Cui, Z., L. F. Lee, J.-L. Liu, and H.-J. Kung. 1991. Structural analysis and transcriptional mapping of the Marek's disease virus gene encoding pp38, an antigen associated with transformed cells. J. Virol. **65**:6509-6515.

Davison, A. J., and J. E. Scott. 1986. The complete DNA sequence of varicella-zoster virus. J. Gen. Virol. **76**:1759-1816.

de Wind, N., A. Zijderveld, K. Glazenburg, A. Gielkens, and A. Berns. 1990. Linker insertion mutagenesis of herpesviruses: inactivation of single genes within the U_S region of pseudorabies virus. J. Virol. **64**:4691-4696.

Dubin, G., E. Socolof, I. Frank, and H. M. Friedman. 1991. Herpes simplex virus type 1 Fc receptor protects infected cells from antibody-dependent cellular cytotoxicity. J. Virol. **65**:7046-7050.

Hanks, S. K., A. M. Quinn, and T. Hunter. 1988. The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains. Science. **241**:42-52.

Holden, V. R., R. R. Yalamanchili, R. N. Harty, and D. J. O'Callaghan. 1992. Identification and characterization of an equine herpesvirus 1 late gene encoding a potential zinc finger. Virology **188**:704-713.

Isfort, R. J., R. A. Stringer, H.-J. Kung, and L. F. Velicer. 1986a. Synthesis, processing, and secretion of the Marek's disease herpesvirus A antigen glycoprotein. J. Virol. **57**:464-474.

Isfort, R. J., I. Sithole, H.-J. Kung, and L. F. Velicer. 1986b. Molecular characterization of Marek's disease herpesvirus B antigen. J. Virol. **59**:411-419.

Johnson, D. C., and V. Feenstra. 1987. Identification of a novel herpes simplex virus type 1-induced glycoprotein which complexes with gE and binds immunoglobulin. J. Virol. **61**:2208-2216.

Johnson, D. C., M. C. Frame, M. W. Ligas, A. M. Cross, and N. D. Stow. 1988. Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral glycoproteins, gE and gI. J. Virol. **62**:1347-1354.

Kimman, T. G., N. de Wind, N. Oei-Lie, J.M.A. Pol, A. J. Berns, and A.L.J. Gielkens. 1992. Contribution of single genes within the unique short region of Aujeszky's disease virus (suid herpesvirus type 1) to virulence, pathogenesis and immunogenicity. J. Gen Virol. **73**:243-251.

Koerner, T. J., J. E. Hill, A. M. Myers, and A. Tzagoloff. 1991. High-expression vectors with multiple cloning sites for construction of *trpE* fusion genes: pATH vectors. Methods Enzymol. **194**:477-490.

Leader, D. P. and F. C. Purves. 1988. The herpesvirus protein kinase: a new departure in protein phosphorylation? Trends Biochem. Sci. **151**:244-246.

Leader, D. P., A. Donella-Deana, F. Marchiori, F. C. Purves, and L. A. Pinna. 1991. Further definition of the substrate specificity of the alphaherpesvirus protein kinase and comparison with protein kinases A and C. Biochim. Biophys. Acta **1091**:426-431.

Lee, G. T.-Y., M. F. Para, and P. G. Spear. 1982. Location of the structural genes for gD and gE and for other polypeptides in the S component of herpes simplex virus type 1 DNA. J. Virol. **43**:41-49.

Lee, L. F., K. Nazerian, S. S. Leinbach, J. M. Reno, and J. A. Boezi. 1976. Effect of phosphonoacetate on Marek's disease virus replication. J. Nat. Cancer Inst. **56**:823-827.

Longnecker, R. and B. Roizman. 1986. Generation of an inverting herpes simplex virus 1 mutant lacking the L-S junction a sequences, an origin of DNA synthesis, and several genes including those specifying glycoprotein E and the a47 gene. J. Virol. **58**:583-591.

McGeoch, D. J., A. Dolan, S. Donald, and F. J. Rixon. 1985. Sequence determination and genetic content of the short unique region in the genome of herpes simplex virus type 1. J. Mol. Biol. **181**:1-13.

McGeoch, D. J. and A. J. Davison. 1986. Alphaherpesviruses possess a gene homologous to the protein kinase gene family of eukaryotes and retroviruses. Nucl. Acids Res. **14**:1765-1777.

McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor. 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69**:1531-1574.

Meignier, B., R. Longnecker, P. Mavromara-Nazos, A. E. Sears, and B. Roizman. 1988. Virulence of and establishment of latency by genetically engineered deletion mutants of herpes simplex virus 1. Virology **162**:251-254.

Nazerian, K., J. J. Solomon, R. L. Witter, and B. R. Burmester. 1968. Studies on the etiology of Marek's disease. II. Finding of a herpesvirus in cell culture. Proc. Soc. Exp. Biol. Med. **127**:177-182.

Nazerian, K. and R. L. Witter. 1970. Cell-free transmission and in vivo replication of Marek's disease virus. J. Virol. **5**:388-397.

Peeters, B., N. de Wind, M. Hooisma, F. Wagenaar, A. Gielkens, and R. Moormann. 1992. Pseudorabies virus envelope glycoproteins gp50 and gII are essential for virus penetration, but only gII is involved in membrane fusion. J. Virol. **66**:894-905.

Pol, J.M.A., F. Wagenaar, and A. Gielkens. 1991. Morphogenesis of three pseudorabies virus strains in porcine nasal mucosa. Intervirology **32**:327-337.

Purves, F. C., D. Spector, and B. Roizman. 1991. The herpes simplex virus 1 protein kinase encoded by the U_S3 gene mediates posttranslational modification of the phosphoprotein encoded by the U_L34 gene. J. Virol. **65**:5757-5764.

Quint, W., A. Gielkens, J. V. Oirschot, A. Berns, and H. T. Cuypers. 1987. Construction and characterization of deletion mutants of pseudorabies virus: a new generation of live vaccines. J. Gen. Virol. **68**:523-534.

Rauh, I. and Mettenleiter, T. C. 1991. Pseudorabies virus glycoproteins gII and gp50 are essential for virus penetration. J. Virol. **65**:5348-5356.

Rixon, F. J. and D. J. McGeoch. 1984. A 3' co-terminal family of mRNAs from the herpes simplex virus type 1 short region: two overlapping reading frames encode unrelated polypeptides one of which has a highly reiterated amino acid sequence. Nucl. Acids Res. **12**:2473-2487.

Roizman, B and A. E. Sears. 1990. Herpes simplex viruses and their replication, p. 1795-1841. *In* B. N. Fields et al. (ed.), Fields virology, vol. 2. Raven Press, New York.

Ross, L.J.N., M. Sanderson, S. D. Scott, M. M. Binns, T. Doel, and B. Milne. 1989. Nucleotide sequence and characterization of the Marek's disease

virus homologue of glycoprotein B of herpes simplex virus. J. Gen. Virol. 70:1789-1804.

Ross, L. J. N. and M. M. Binns. 1991. Properties and evolutionary relationships of the Marek's disease virus homologues of protein kinase, glycoprotein D and glycoprotein I of herpes simplex virus. J. Gen. Virol. 72:939-947.

Ross, L. J. N., M. M. Binns, and J. Pastorek. 1991. DNA sequence and organization of genes in a 5.5 kbp Eco RI fragment mapping in the unique short segment of Marek's disease virus (strain RB1B). J Gen. Virol. 72:949-954.

Schultz, A. and S. Oroszlan. 1984. Myristilation of *gag-onc* fusion proteins in mammalian transforming retroviruses. Virology 133:431-437.

Scott, S. D., N.L.J. Ross, and M. M. Binns. 1989. Nucleotide and predicted amino acid sequences of the Marek's disease virus and turkey herpesvirus thymidine kinase genes; comparison with thymidine kinase genes of other herpesviruses. J. Gen. Virol. 70:3055-3065.

Serafini-Cessi, F., F. Dall'Olio, M. Scannavini, and G. Campadelli-Fiume. 1983. Processing of herpes simplex virus-1 glycans in cells defective in glycosyl transferases of the Golgi system: relationship to cell fusion and virion egress. Virology. 131:59-70.

Simek, S. and N. R. Rice. 1988. $p59^{v\text{-}rel}$. the transforming protein of reticuloendotheliosis virus, is complexed with at least four other proteins in transformed lymphoid cells. J. Virol. 62:4730-4736.

Solomon, J. J. 1975. Preparation of avian cell culture. Tissue Culture Methods 1:7-11.

Spring, S. B., B. Roizman, and J. Schwartz. 1968. Herpes simplex virus products in productive and abortive infection. II. Electron microscopic and immunological evidence for failure of virus envelopment as a cause of abortive infection. J. Virol. 2:384-392.

Telford, E.A.R., M. S. Watson, K. McBride, and A. J. Davison. 1992. The DNA sequence of equine herpesvirus-1. Virology 189:304-316.

Towler, D. A., S. R. Eubanks, D. S. Towery, S. P. Adams, and L. Glaser. 1987. Amino-terminal processing of proteins by *N*-myristoylation. Substrate specificity of N-myristoyl transferase. J. Biol. Chem. 262:1030-1036.

Umene, K. 1986. Conversion of a fraction of the unique sequence to part of the inverted repeats in the S component of the herpes simplex virus type 1 genome. J. Gen. Virol. 67:1035-1048.

Vafai, A., Z. Wroblewska, R. Mahalingam, G. Cabirac, M. Wellish, M. Cisco, and D. Gilden. 1988b. Recognition of similar epitopes on varicella-zoster virus gpI and gpIV by monoclonal antibodies. J. Virol. 62:2544-2551.

Vafai, A., K. Jensen, and R. Kubo. 1989. Existence of similar antigenic-sites on varicella-zoster virus gpI and gpIV. Virus Res. 13:319-336.

van Zijl, M., H. van der Gulden, N. de Wind, A. Gielkens, and A. Berns. 1990. Identification of two genes in the unique short region of pseudorabies virus; comparison with herpes simplex virus and varicella-zoster virus. J. Gen Virol. 71:1747-1755.

Weber, P. C., M. Levine, and J. C. Glorioso. 1987. Rapid identification of nonessential genes of herpes simplex virus type 1 by Tn5 mutagenesis. Science 236:576-579.

Witte, O. N. and D. F. Wirth. 1979. Structure of the murine leukemia virus envelope glycoproteins precursor. J. Virol. 29:735-743.

Zhang, G. and D. P. Leader. 1990. The structure of the pseudorabies virus genome at the end of the inverted repeat sequences proximal to the junction with the short unique region. J. Gen. Virol. 71:2433-2441.

Zsak, L., F. Zuckermann, N. Sugg, and T. Ben-Porat. 1992. Glycoprotein gI of pseudorabies virus promotes cell fusion and virus spread via direct cell-to-cell transmission. J. Virol. **66**:2316-2325.

Zuckermann, F., T. Mettenleiter, C. Schreurs, N. Sugg, and T. Ben-Porat. 1988. Complex between glycoproteins gI and gp63 of pseudorabies virus: its effect on virus replication. J. Virol. **62**:4622-4626.

Chapter V

Summary and Conclusions

One of the original goals of this project was to identify new glycoprotein genes potentially important in protective immunity. The identification and characterization of MDV genes and/or their products homologous to HSV gD, gI, and gE fullfilled this initial goal. However, the sequence of events leading to this finding had an important impact on the subsequent evolution of this project.

Soon after MDV was classified as a gammaherpesvirus (based on biological properties) its genome structure was found to be essentially identical to that of the alphaherpesvirus prototype, HSV. Published findings at that time identified a similarly conserved glycoprotein gene cluster located in the U_S regions of the HSV and PRV. Recognition of this point raised the question of whether MDV might contain a similar glycoprotein gene cluster. The objective of my work was to identify whether such genes existed in the U_S region of MDV. A transcriptional mapping approach was initiated to seek out such genes. The idea was to identify abundantly expressed, PAA-sensitive transcripts characteristic of glycoprotein genes. A transcript matching these criteria was soon found. S1 nuclease protection analysis mapped the precise location of the transcript; this facilitated the nucleotide sequence determination of its corresponding DNA segment. This took place at a time when nucleotide sequencing of MDV had just begun (when I started my work, no MDV genes had yet been sequenced). To our surprise, the region encoding the 1.9 kb EcoRI-O transcript was found to contain two ORFs homologous to the alphaherpesvirus-specific products of HSV US1 and -10. At about the same time these results were obtained, Buckmaster and others associated with the laboratory of Norman Ross (1988) published the first direct evidence indicating a closer phylogenetic relationship between MDV and alphaherpesviruses. These findings raised an important fundamental question. What is

the genetic basis for an alphaherpesvirus having biological properties common to gammaherpesviruses?

Two observations suggested that a further analysis of the MDV U_S region might lead to a better understanding of this apparent discrepancy. U_S- (and other S region) genes derive from an area exhibiting significant genetic divergence and containing a cluster of *supplementary essential* genes, whose functions are non-essential for growth in cell culture. These genes are thought to specify biological properties characterizing different herpesviruses from one another (see Chapter I). In addition to addressing the nature of the classification discrepancy above, MDV's close relationship with alphaherpesviruses offered potentially new experimental approaches based on information about MDV U_S homologs in better characterized alphaherpesvirus systems. This information offered to promote rapid progress in our understanding of the molecular biology of MDV. This newly recognized potential underscored our need to identify and characterize new MDV genes (a process that had just begun). In light of these factors my analysis of the MDV U_S region took on a broader scope. As a result, the complete nucleotide sequence of MDV's U_S region was determined. This 11,160-bp segment was found to contain 7 alphaherpesvirus-related genes, 3-non-herpesvirus-related genes and 1 fowlpox virus related gene. In conjuction with this work, the location of the U_S-inverted repeat junction sites were precisely identified, correcting previous results which suggested a larger U_S region.

Determination of the nucleotide sequence allowed for the construction of plasmid clones to direct the inducible expression (in E. coli) of fusion protein immunogens to facilitate generation of antibodies with specificities for U_S region polypeptides. Antibodies generated from a pool of 16 different fusion protein immunogens (corresponding to 9 of the 11 U_S ORFs) allowed for the identification and/or immunoprecipitation of 6 of the 7 alphaherpesvirus-related MDV U_S region products from infected DEF cultures.

Although graduate research projects have traditionally involved a one or two gene characterization approach (which tends to allow for a timelier exit), in defense of this project, it should be emphasized that the characterization of nucleotide sequences and polypeptides represents one of the more pressing needs in the MDV system. Unlike better characterized systems (such as HSV), MDV workers are only beginning to lay a foundation for molecular biological studies. Notwithstanding the results presented here, a survey of the current literature has identified just 4 MDV genes whose nucleotide sequences and polypeptides have been characterized.

The antibody reagents generated from this study led to a number of interesting findings that should pave the way for a number of important studies. In the course of my work I have paid particular attention to an unusual 27-kDa late class cytoplasmic phosphoprotein homologous to the 68-kDa HSV-1 immediate early nuclear phosphoprotein, ICP22. Since ICP22 homologs appear to represent a cell tropism-determining regulatory protein, further studies involving pp27,24 may shed light on the question of lymphotropism vs. neurotropism. In light of the paucity of information regarding ICP22 homologs and lymphotropism among alphaherpesviruses, it might be worthwhile to examine whether ICP22 homologs promote growth in lymphocytes.

Non-reducing conditions enhanced the identification of a band approximately double the size of pp27,24. This suggests that pp27,24 forms dimers under native conditions. Not only is this property consistent with a potential role in DNA-binding, but it raises the possibility that ICP22 homologs manifest their regulatory, cell-tropism-determining nature by facilitating various types of protein-protein interactions. In contrast to other ICP22 homologs, pp27,24 appears to lack proline-associated basic regions associated with nuclear localization signals. Perhaps its function has evolved to effect the cytoplasmic retention of other nuclear transcription factors by way of its ability to mediate protein-protein interactions. This is a familiar feature which

characterizes the Rel family of proteins. The generation of pp27,24-specific antisera presents an opportunity to explore these possibilities.

The biologic- vs. genetic property "discrepancy" could be further addressed by analyzing the role of non-alphaherpesvirus-related U_S region genes (e.g. SORFs 1-4 of Chapter II). SORF3, located in the EcoRI-O subfragment, specifies a 351 aa MDV-specific ORF. Northern blot experiments employing probes identifying US1 transcripts failed to identify transcripts likely to code for SORF3. However, previously reported data suggest that a gene in EcoRI-O is expressed in MDV tumor cell lines (Schat et al., 1989). Could it be that SORF3 exhibits lymphoid-specific gene expression? Such a possibility could account for the failure to detect SORF3 polypeptides using trpE-SORF3-directed antibodies. If the ecological niche concept of Roizman and colleagues is correct, then it follows that certain MDV U_S region genes are likely to be expressed in lymphoid cells, possibly in a cell-specific manner.

Considering the properties associated with U_S region glycoproteins related to HSV (e.g. gD, -I and -E), a number of potentially interesting MDV studies can be addressed. In addition to their importance in pathogenesis and immunoprotection, glycoproteins present an excellent opportunity for studying virus-cell interactions (such as lymphotropic vs. neurotropic growth etc.). Here the MDV system is particularly attractive. Chickens provide a useful experimental background amenable to examination of these interactions as they occur in nature. Phenotypic consequences associated with the use of genetically engineered mutant MDV strains can be studied at will. Of particular interest is the role (or non-role) of gD. The evidence presented appears to suggest that MDV's gD counterpart may not be expressed in vitro or in vivo. As outlined in Chapter IV, such a situation may represent a useful adaptation benefiting the dissemination and persistence of MDV. Despite its essentiality for HSV, VZV has apparently benefited from the absence of such a glycoprotein. Further MDV studies are clearly warranted to resolve these findings and determine their significance

in relation to the life cycle of MDV. Characterization of the MDV gI/gE homologs in this (and in an accompanying) study should lead to a further examination of their contribution to dissemination and pathogenesis. Considering that alphaherpesvirus gEs appear to have a role associated with cell-cell spread it would be of interest to examine the phenotypic consequences associated with MDV gE mutants. Since MDV is strongly cell-associated, MDV's gE may encode a glycoprotein whose function is indispensable for growth. Similar questions should extend to other U_S region genes as well.

Generally speaking, alphaherpesvirus S region genes encode late-regulated and/or virion-associated products (see chapter I). Such functions may facilitate viral growth and dissemination by providing a source of products operative immediately upon entry into cells. In this way late products more closely resemble what is tantamount to an "immediate-immediate-early" product, effectively providing the virus with a head-start against the counter (immune) mechanisms responsive to the initial infection and the subsequent viral gene expression that follows. The presence of late gene, virion-associated functions would be consistent with presence of functions conferring optimal growth and dissemination (as proposed by Roizman and colleagues). Preliminary experiments indicate that at least 5 of MDV's U_S region genes (US1, -3, -7, -8, and 10) are regulated as late genes (expression inhibited by PAA). The antisera generated should allow for an examination (by western blot analysis) of their possible association with purified virions. For US1, such a possibility could reflect a novel negative regulatory strategy opposite to that involving HSV VP16. Since other data suggests a possible role for ICP22 homologs in negative regulation (see Chapters 1,3), by extension pp27,24 might counter the onset of fully productive infections. Such a possibility would be consistent with recent findings indicating a lack of US1 expression in feather follicle epithelial cells (R. Witter, pers. comm.).

Precipitation of the 68-kDa cellular protein with antisera directed against MDV US3 (PK) presents another potentially interesting direction for further studies. Although alphaherpesvirus PK-related cellular genes are thought to exist, none have been found thus far. Screening of lambda-gt11 expression libraries with this antisera may allow for the identification of such homologs.

The work I have accomplished is more representative of a beginning than an end. Determination of the U_S region nucleotide sequence has located genes whose expression can be more accurately defined using specific nucleic acid probes. Similarly, the availability of new antisera should permit further characterization of MDV U_S region gene expression at the polypeptide level. It is hoped that the information and reagents generated from this study will be of use in resolving many of the questions and possibilities put forth in this thesis.