Molecular Evolution of Viruses – Past and Present

Evolution of Viruses by Acquisition of Cellular RNA and DNA

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PART A:

Virus Genes Acquired to Evade the Host Immune Responses

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Evolution of Viruses by Acquisition of Cellular RNA or DNA Nucleotide Sequences and Genes: An Introduction

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Abstract. The origins of virus evolution may be traced to Archeabacteria since Inouye and Inouye (6) discovered a retroelement with a gene for reverse transcriptase in the bacterial genome and in the satellite, multiple copy single stranded DNA (msDNA) in the soil bacterium Myxococcus xanthus. It was possible (8) to define the evolution of retroelements in eukaryotic cells of plants, insects (gypsy retrovirus) and vertebrates. The replication of RNA viruses in eukaryotic cells allowed for the viral RNA genome to integrate a cellular ubiquitin mRNA, as reported for BVDV (24). Another example is the integration of 28S ribosomal RNA into the hemagglutinin gene of an influenza virus. This change in the hemagglutinin gene led to an increased pathogenicity of the influenza virus (25). In contrast to RNA viruses, DNA viruses had evolved by inserting cDNA molecules derived from mRNA transcripts of cellular genes or foreign viral RNA. It is of interest that the virus acquired cellular genes in the genomes of DNA viruses represent genes that code for proteins that inhibit cellular molecular processes related to HLA class I and II molecules. The other acquired genes are cellular genes that code for cytokines that are capable of inhibiting antigen presentation to T cells by antigen presenting cells (APC) by dendritic Langerhans cells. The acquisition of cellular genes by DNA viruses enhances their pathogenicity by inhibiting the hosts' defense systems.

Key words: virus evolution, retroviral evolution, evolution of eukaryotic cells, virus acquired cellular genes

Introduction

The two special issues of Virus Genes (1–2) were dedicated to studies on the origin and evolution of RNA and DNA viruses that infect prokaryotic archeabacteria and eubacteria, algae, fungi, insects and vertebrates including humans. Attention was given to the evolution of retron and retrotransposons during speciation and to the acquisition of the gene that codes for the envelope protein by the Drosophila Gypsy retrotransposon. The present special issue is devoted to the current concepts on the early evolution of the eukaryotic cells and to the identification of cellular genes and nucleotide sequences that may have been acquired by RNA and DNA viruses.

The molecular events that led to the appearance of viruses in prokaryotic and eukaryotic cells and their evolution during a period of two billion years is an unsolved enigma. In collaboration with molecular virologists interested in virus evolution, it is hoped that the ongoing advancements in the deciphering of the genes in genomes of ancient prokaryotes, eubacteria and eukaryotes it will be possible to trace the evolution of fungi, insects, vertebrates and man. In parallel to the evolution of the species, viruses had evolved by capturing and using genes from the infected cells for functions that they require to produce their progeny, to enhance their escape from the host cellular and humoral immune systems and to cope with the intracellular and extracellular environments. It may be possible to envision that the early RNA and DNA viruses functioned as sets of genes capable of self replication in prokaryotic and eukaryotic infected cells by using a cellular polymerase gene (e.g. reverse transcriptase of the archeabacterial retron (4)). The viral genomes had continued to evolve during the evolution of the eukaryotic species.

One billion years, from 4.5 to 3.5 billion years ago, were required for nature to develop from the

hypothetical ''prebiotic soup'' that led to the appearance of RNA molecules (the ''RNA world'' defined by (3) as ''evolution of life'' (4)) and to develop primitive membranes and cells, until cyanobacteria evolved. Woese and Pace (5) considered RNA to be a historical record and used ribosomal RNA for a quantitative analysis of evolutionary relationships between cyanobacteria that are 3.65 billion years old and Archeaebacteria and Bacteria which preceded the evolution of eukaryotic cells (Eucarya).

Inouye and Inouye (6) discovered the structure and function of an Archeabacterial reverse transcriptase gene that is present in a retroelement form as a satellite multiple copy single-stranded DNA (msDNA) isolated from Myxococcus xanthus, a gram negative soil bacterium. This retron, that is present as a locus in the prokaryotic bacterial chromosome, contains the genes for msrd, msr and the gene for reverse transcriptase (6). It was indicated by the authors that the msDNA molecules are found in all seven genera of the myxococcus subgroup. While 95% of the Myxobacteria contain the retron with the reverse transcriptase, only 10% of E. coli, 5% of Klebsiella, 17% of Proteus mirabillis, 6% of Salmonella, 16% of Rhizobia and Bradyrhizobia were found to contain this retron (7). The understanding that the reverse transcriptase gene-containing retrons had evolved before or during the evolution of Archeabacteria (6) was complemented by the analysis (8) on the evolution of retroelements that are present in eukaryotic cells of plants, insects and higher organisms: CaMV (cauliflower mosaic caulimovirus), CoYMV (Commelina yellow mottle badnavirus), Ty (yeast Ty retrotransposon), Gypsy (Drosophila Gypsy retrotransposon) and Copia (Drosophila copia retrotransposon). Hull and Covey concluded that most retroelements have additional genes with regulatory or adaptive roles, both within the cell and for movement between cells and organisms. It is possible to trace the acquisition of a gene that codes for the envelope protein (env gene) that transforms a retrotransposon into a retrovirus to the gypsy retroelement of Drosophila, since it had acquired, most probably from the insect cells, the envelope gene that allows the assembly and release of infectious virions. The studies on the gypsy retrotransposon had bridged the gap in the understanding of the evolutionary pathway of the Archeabacterial reverse transcriptase gene (6) that existed in nature in Archeabacteria and contributed to the reverse transcriptase of retroviruses of vertebrate (9). Brosius and Tiedge (10) concluded that the reverse transcriptase gene is a mediator of genomic plasticity stating that ''reverse transcriptase, an enzyme whose cellular function is still enigmatic, may still exert—as it did more than three billion years ago when the RNA genome was converted in the DNA genome—a great influence on genomic plasticity, by not only providing novel genes but also mixing existing genes with novel regulatory elements. Altering when, where and how much a gene is expressed can have great evolutionary impacts'' (10).

In the introductions to the Virus Genes Special Issues on Virus Evolution I had attempted to draw attention to the experiments and theories on the ''RNA world'' that had evolved to a ''DNA world'' when the prokaryotic and eukaryotic cells appeared three billion years ago (4,11). The current advances in the sequencing of the DNA genomes of prokaryotes and eukaryotes made it possible to identify the bacterial genes that had been involved in the evolution of the nuclear DNA genomes of eukaryotic cells and their chloroplasts (in plant cells) or mitochondria (in animal cells). In the present issue, studies are presented that identify genes in RNA and DNA viruses that may have been acquired from the nuclear DNA genome of the infected eukaryotic cells. Attention is given to current understanding of the evolution of the eukaryotic cells (12).

Evolution of the Eukaryotic Cell

Gupta and Golding in their reviews (12) listed earlier theories on the evolution of eukaryotic cells that were started with the suggestion by Mereschkowsky in 1905 on the symbiotic origin of eukaryotic cells to the concept that the eukaryotic nuclear genome evolved from an archeabacterial ancestor (13–16), the currently favored view. Gupta (17) pointed that the recognition of archeabacteria by (15) as a distinct life form and ancestors of eukaryotic cells had lead to further analysis and comparisons of common genes. Gupta et al. (18) cloned two genes coding for two different 70 KDa heat shock protein (GSP70) homologs from the protozoan Giardia Lamblia, which lacks mitochondria (19) and constitutes the earliest divergent member within the eukaryotc lineage on the basis of 16S rRNA phylogeny (20). One of these Giardia genes is the cytoplasmic form of HSP70 and the

second gene is the endoplasmic reticulum (ER)—resident heat shock protein GRP78, a member of the Bip family. The identification of an ER resident GRP78 protein in G. lamblia, a primitive eukaryotic ''archazoan'' that lacks mitochondria and other organelles, strongly suggests the existence of ER in this ancient eukaryote. Phylogenetic analyzes of HSP70 sequences showed that the cytoplasmic and ER homolog form distinct subfamilies that evolved from a common eukaryotic ancestor by gene duplication that occurred very early in the evolution of eukaryotic cells (18). In a more detailed analysis Gupta et al. (18) suggested that ''diderm prokaryotes'' (i.e. gram-negative bacteria), which have a bilayered cell wall are derived from monoderm prokaryotes (gram positive bacteria) with a single-layered cell wall (e.g. Archeabacteria). The authors hypothesized that ''all eukaryotes'', including amitochondria and aplastidic organisms, received major gene contributions from both archeabacterium and a gram negative bacteria, evolving into the ancestral eukaryotic cell with a genome resulting from the DNA genomes of two separate prokaryotes. Gupta et al. (18) proposed that the eukaryotic cell nucleus started as a symbiotic association between a gram negative bacterium and proteobacteria (e.g. Dinococcus thermus, Cyanobacteria, Chlamydia cytophaga and two different groups of proteobacteria engulfing an eocyte (e.g. Archeabacterium). The disappearance of the Archeabacterium membrane and the infolding of the proteobacterium cell membranes resulted in the formation of the membranes of the endoplasmic reticulum and the nuclear membrane. The inclusion of both DNA genomes in the nucleus led to the appearance of the ancestral eukaryotic cells (12,17).

Although the above model for the evolution of eukaryotes is based on a limited number of gene homologs, it should be noted that if the Archeabacterial genome was involved in the evolution of the eukaryotic cells it contributed the reverse transcriptase and the msDNA genes to the primitive eukaryotes. The reverse transcriptase gene had a role in the further evolution of the nuclear DNA genome of the ancestral eukaryotic cells as suggested by (8) and (10). With the inclusion of the reverse transcriptase gene in the developing pre-eukaryotic cells, the genome could have been expanded by the generation of DNA copies from mRNAs, small nuclear and cytoplasmic RNAs resulting in reinsertion of the DNA molecules as genes and short interspersed repetitive elements (SINEs). The repetitive DNA sequences in eukaryotic genomes are thought to reflect the evolutionary forces acting on selfish DNA (10). It may be possible to suggest that the Archeabacterial retrons (6) further evolved, by recruiting essential cellular genes, into RNA and DNA viruses.

In a recent review on mitochondrial evolution (21) Gray et al. indicated that gene sequence data suggest that the mitochondrion ''arose in a common ancestor of all extant eukaryotes raising the possibility that this organelle originated at essentially the same time as the nuclear component of the eukaryotic cell''.

Endogenous retroviruses in sharks, bony fish, reptiles, birds and mammals. Hull and Covey (8) suggested that retroelements that comprise a gag-pol replicon core to which adaptive genes were added (e.g. envelope gene) adapted to behave as ''selfish nucleic acids''. To screen for the presence of endogenous retroviruses within the genomes of 18 vertebrate orders across eight classes concentrating on reptilian, amphibian and piscine hosts, Herinou et al. (22) used the PCR test on DNA samples that were obtained from more than 50 taxa that included members of eight vertebrates and three nonvertebrate classes. The DNA samples were derived from reptiles, amphibians and bony fish using degenerate primers from reverse transcriptase and the protease genes. The authors reported that they were unable to identify retroviral sequences in nine species of molluscs, tunicates, lancelets (the sea squid *Ciona intestinalis* from the order Urochordata), Hag fish (Taiwanese hagfish Myxine yangi) and Lampreys (the river lamprey Lampetra fluviatilis). Endogenous retroviruses were identified in sharks, bony fish, amphibians, reptiles, birds and mammals (22).

C. Leib-Mosch and Seigurth (23) studied the evolution and biological significance of three types of human retroelements: the endogenous retroviruses (e.g. HERV-K), retrotransposons (e.g. RTVL-H human THE-1) and nonviral retroposons (e.g. LINE-1, SINE-ALU and SINE-R). It will be of interest to know if the nonvertebrates that lacked endogenous retroviruses still contained nonviral retroposons.

Evolution of RNA viruses by acquisition of RNA molecules from the host cells: ubiquitin and ribosomal RNA genes. Meyer et al. (24) reported that isolates of bovine viral diarrhea virus (e.g. BVDV osloss

isolate) with a single stranded RNA genome (genus pestivirus, family Togaviruses) was found to contain an insert of 228 nucleotides in a region coding for a viral nonstructural protein. The insert encodes a complete ubiquitin-like element with two amino acid exchanges with respect to the ubiquitin sequence conserved in all animals. Another BVDV CP1 isolate RNA genome was found to contain one and a half ubiquitin genes. In contrast, the isolate, BVDV NADL genome contains a 270 nucleotide insert that showed no homology to a ubiquitin gene but is almost identical with another bovine mRNA sequence. Since pestiviruses replicate in the cytoplasm of infected cells, the integration of RNA sequences into the viral RNA genome had occurred with the help of cellular enzymes that are involved in RNA recombination. The ubiquitin gene containing BVDV isolates are cytopathogenic in infected cells while the original virus is noncytopathogenic. The authors quote the study by Khatachikian et al. (25) reporting on an insertion of a 28S ribosomal RNA sequence into the hemagglutinin gene of an influenza virus that increased the pathogenicity of the virus. These studies may be taken to indicate that RNA viruses can incorporate cellular mRNAs into their viral genome by RNA recombinational events. The foreign viral or cellular genes may enhance virus pathogenicity.

Evolution of DNA viruses by acquisition of cellular DNA sequences. Epstein-Barr virus (EBV) DNA genome contains the EBFA-1 gene with a 708 bp nucleotide sequence that is made of three nucleotide triplets GGG, GGA and GCA (designated IR3 repetitive region) encoding a repetitive glycine-alanine domain of the viral protein EBNA-1. Heller et al. (26) isolated and cloned human and mouse cellular DNA that cross-hybridized with the IE3 repetitive sequence of the EBNA-1 gene. The authors reported that monoclonal antibodies raised against glycine-alanine domains of EBNA-1 also recognized a cellular protein in uninfected lymphocytes. It was suggested that the amino acids GGA repeats may be ubiquitous in eukaryotic genomes and are present as long nucleotide arrays.

Kishi et al. (27) reported that a repeat sequence GGGTTA is present in the DNA of human herpesviruses—6 (HHV6), HHV-7 and in the DNA genome of the chicken Marek's Disease virus (MDV). The repeat sequence GGGTTA is present in the highly conserved repetitive DNA sequence present in the telomeres of human chromosomes as a defined region of the DNA at the molecular end of a linear chromosomal DNA that is required for the replication and the stability of the chromosome (28). Meyne et al. (29) determined the evolutionary origin of the human telomeric sequence $(TTAGGG)_n$ in 91 different species. A biotinylated oligonucleotide of this sequence was used for hybridization to metaphase chromosomes from cells of bony fish (evolved 408 million years ago), amphibians (350 million years ago), reptiles (330 million years ago), birds (210 million years ago) and mammals (220 million years ago). The chromosal telomeres were detected in all 91 species. The function and role of the cellular telomeric sequence that is present in MDV, HHV-6 and HHV-7 is still to be determined.

The current advancements in sequencing of prokaryotic and eukaryotic cellular and viral genomes provide information on the properties of the genes and the proteins coded by them. These developments lead to the organization of the information in gene and protein banks, and nucleotide or amino acid sequences of viral and cellular genes are identified by their accession numbers. Therefore, computer programs that allows rapid homology analysis between genes of different viruses that was developed by Altschul et al. (30), the gapped BLAST and PSI-BLAST, allow rapid comparison of genes and protein data banks. These computer programs allow the comparison between viral genes and proteins to cellular genes and proteins helping to trace the evolutionary pathways of virus genes.

Evolution of DNA viruses by acquisition of nuclear genes from the infected cells. Acquisition of ubiquitin and ubiquitin conjugating genes by the DNA virus, by the Baculovirus Autographa californica nuclear polyhydrosis virus (ACMNPV) was studied by Giarnono et al. (31). The virus encodes a protein, v-ubi, that has 76% identity with the eukaryotic protein ubiquitin. The v-ubi gene was transcribed during the late phase of virus replication. The v-ubi gene was identical to a polyubiquitin gene that was isolated form Spodoptera frugperda, a lepidopteran cell line, the host cell for ACMNPV.

African swine fever virus (ASFV) DNA genome was reported to encode ubiquitin conjugating enzyme which is similar to the yeast ubiquitin conjugating enzymes UBC2 and UBC3. (32,33).

The ability of DNA viruses to acquire cellular

genes indicate that these viruses are able to interfere with the cellular control of programmed degradation of cellular proteins by the cytoplasmic proteasomes. These acquired virus genes that function in the virus infected cells under the control of virus specified promoters provide the infecting virus with an advantage over the host cell. It will be important to explore the properties of the promoters of the cellular genes that were incorporated in the viral genomes.

The DNA genome of the lymphotropic Herpesvirus saimiri (HVS) that was isolated from squirrel monkeys was sequenced by Albrecht et al. (34) who identified 8 ORFs that are homologous to cellular genes: ORF 01 (designated STP-A) contains collagen repeats; ORF 02 (designated DHFR) is 83% identical to human dehydrofolate reductase (DHFR); ORF 04 a/b is a complement control protein homolog; ORF14 (designated IEG) a possible superantigen gene; ORF15 homologous to human CD59 (protectin-resticts the cytolytic activity of homologous complement); ORF 70 (designated TS—thymidine synthase) 66% identity to human TS; ORF 72 (designated ECLF2) has 25% identity to human cyclin D; ORF 74 (designated ECRF3) has 30% identity to human IL-8 receptor.

These studies (34) indicated that a lymphotropic DNA virus of monkeys had acquired cellular genes that are essential for the virus to control molecular processes of the infected cell.

The monkey lymphotropic virus HVS has a genome organization consisting of a unique DNA sequence flanked by two repeat sequences. This genome organization resembles the genome of channel catfish herpesvirus, the human lymphotropic Epstein-Barr Virus (EBV) and the lymphotropic human Herpesviruses 6,7 and 8 (HHV-6, HHV-7, HHV-8). In contrast, the DNA genome organization of the chicken Marek's Disease Virus (MDV) is organized in two unique DNA sequences, one long (L) and the second short (S), that are flanked by repeat sequences. The genome organization of MDV-infected chickens (evolved $\sim$ 170 million years ago) differed from the DNA genome of the channel catfish herpesvirus infecting bony fish (evolved 70 million years ago) that resembles the genome organization of HVS. It is not yet concluded whether the ancestral herpesvirus contained a genome with one Unique Long sequence or a genome with the Unique Long and Unique Short DNA sequences.

Acquisition by DNA Viruses of host cellular genes that allow these viruses to counteract the immune system of the vertebrate host. The present Special Issue provides the current knowledge on virus genes that were acquired from the eukaryotic host cells probably as a protection from the defence mechanisms of the infected cells of the host organisms. Alternatively, it is possible that the evolution of pathogenic viruses had led to improvements in the development of eukaryotic genes of the immune systems during the 500 million years of evolution of the bone marrow in bony fish, amphibians, reptiles, birds and mammals.

Three examples of viruses that code for viral proteins capable of interfering with the mammalian host immune systems are presented: The herpesvirus cytomegalovirus, adenovirus and hepatitis B virus (Part A).

The characterization of genes in the DNA genomes of Marek's Disease Virus (MDV), human herpesviruses and iridoviruses as homologs of eukaryotic cellular genes (Part B) provide the identification of acquired virus genes that include genes coding for chemokines and chemokine receptors, cytokine homologs, apoptosis regulating genes, major histocompatibility (MHC) complex homologs, complement-regulating protein homologs, Fc-receptor homologs, and genes that code for immunoglobulin-superfamily proteins. It can be seen that the DNA genomes of the human herpesviruses contain almost a complete set of genes for proteins that regulate the activities of the immune cells. One example is the gene for IL-10 that was acquired by several herpesviruses (e.g. EBV). Induction of the synthesis of the viral gene coding for IL-10 in epidermal cells of the nasopharynx during an infection by EBV will inactivate the Langerhans cells, the most important antigen presenting cells in the vertebrate host, and thus prevent or delay the antiviral cellular immune response and the induction of antiviral cytotoxic T cell response (35). Additional studies will be needed to identify the ways by which the acquired cellular genes are used for the benefit of the infecting virus. Another example (Part B) is the acquisition of a cellular gene by Marek's Disease Virus (MDV) a transforming chicken virus that is a homolog of the host cell c-Jun oncogene.

The availability of the complete nucleotide sequences of the DNAs from human poxviruses made it possible to identify virus gene homologs of cellular genes that were acquired by poxviruses from

their hosts during virus evolution (e.g. IL-10 gene of the parapoxvirus Orf virus and the TNF Receptor gene homolog of myxoma virus). A summary of the known poxvirus gene homologs of cellular genes present in the poxvirus DNA genomes is also presented (Part C).

An interim summary. The three special issues on virus evolution attempted to provide information on the past and present molecular events that shaped the evolution of RNA and DNA viruses. The advances in the international efforts to sequence the genomes of ancient prokaryotic and eukaryotic cells and all virus genomes coupled with the developments in computer programming allow now to search for the origins of virus genes homologs of cellular genes. From this information it is possible to note that RNA and DNA viruses had used the host cell molecular processes that are involved in nucleic acid recombination to acquire cellular genes that may help them control the infected cells and the host organism's immune responses.

In the future special issues of Virus Genes on the Origin and Evolution of Viruses, we will look into the molecular mechanisms by which viruses use their genes to escape from the host's defense mechanisms in vertebrates and the origin of the virus genes that code for the viral structural and regulatory proteins. Analysis of viruses that had evolved during the last three billion years may lead us to solve the enigma of the origin of RNA and DNA viruses.

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Immunomodulatory Functions Encoded by the E3 Transcription Unit of Adenoviruses

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Abstract. Persistent viruses have evolved multiple strategies to escape the host immune system. One important prerequisite for efficient viral reproduction in the face of an ongoing immune response is prevention of premature lysis of infected cells. A number of viruses achieve this goal by interfering with antigen presentation and recognition of infected cells by cytotoxic T cells (CTL). Another viral strategy aims to block apoptosis triggered by host defense mechanisms. Both types of strategies seem to be realized by human adenoviruses (Ads). The early transcription unit E3 of Ads encodes proteins that inhibit antigen presentation by MHC class I molecules as well as apoptosis induced by tumor necrosis factor α (TNF-α) and Fas ligand (FasL). Here, we will describe the organization of the E3 regions of different Ad subgroups and compare the structure and functions of the known immunomodulatory E3 proteins.

Key words: adenovirus E3 proteins, E3 protein sequence comparison, immune evasion, interference with antigen presentation, CD95 (Fas/APO-1), apoptosis, receptor down-regulation, TNF mediated lysis

Introduction

Adenoviruses (Ads) are non-enveloped viruses of eicosahedral structure. The virion contains a double-stranded DNA genome of approximately 36000 bp. At least 47 different human Ad serotypes have been described which are classified into 6 subgroups A-F, according to their DNA homology and some other criteria, such as their oncogenicity in newborn rodents (1).

Human Ads cause acute as well as persistent infections (2). A wide range of mostly mild diseases are associated with these infections. Although each Ad subtype can infect a great variety of tissues and cells, a distinct disease pattern is observed for Ads belonging to different subgroups: Ads of subgroup A (e.g. Ad12) and F (Ad40, Ad41) cause gastrointestinal infections, the latter primarily in infants, whereas Ads of subgenus B and C are mainly associated with infections of the upper respiratory tract, which may be accompanied by acute respiratory disease, pertussis like syndrome or pneumonia (2). Subgenus B viruses tend to cause more severe respiratory disease whereas serotypes of subgroup C frequently establish persistent infections of lymphoid tissues (2). Ad34 and Ad35 of subgroup B2 have the propensity to persist in the urinary tract and are commonly isolated from immunosuppressed patients (bone marrow transplants, AIDS patients), while Ad4 (subgroup E) causes respiratory infections. Subgroup D harbours more than half of all adenovirus subtypes, including Ad8, Ad19 and Ad37 which are specifically associated with a highly contagious and relatively severe eye disease, called epidemic keratoconjunctivitis (EKC).

A key role for regulating the interaction of Ads with their host has been attributed to proteins encoded in the non-essential early transcription unit 3 (E3) of the virus (3,4). Firstly, none of the E3 proteins is required for replication of the virus *in vitro* and *in vivo* in cotton rat lungs (5). Secondly, several E3 proteins have been reported to counteract host defense mechanisms: The E3/19K protein interferes with antigen presentation and T cell recognition, for

review (4), while the E3/14.7K, 10.4K and 14.5K proteins can protect cells from TNF-mediated and Fas ligand mediated apoptosis (3,6–9). The aim of this review is to compare the organization of the E3 regions in Ads of different subgenera, and to summarize the known immunomodulatory functions of E3 proteins. Furthermore, we will discuss the structural relationship and the relative conservation of the individual immunomodulatory E3 proteins, taking into account the new sequence information provided for the E3/10.4K, 14.5K and 14.7K proteins of subgroup D Ads.

Organization of the Early Transcription Unit 3 (E3) in Adenoviruses Belonging to Different Subgroups

The size and composition of the E3 transcription unit is not conserved but varies considerably between Ads from different subgroups. In subgroup F (Ad40/Ad41) the E3 region encompasses approximately 3000 bp whereas it is enlarged to about 5200 bp in subgroup D (Ad19a). The variation in size is due to the different number and the different coding capacity of the individual genes present in the respective E3 regions (Fig. 1). While, for example, subgroup F has only five open reading frames (ORFs), subgroup D has eight and subgenus B1 (Ad3, Ad7) even 9 ORFs larger than 8 kDa (3,10,11). Some of these genes, like those encoding 10.4K, 14.5K and 14.7K (black bars), exist in all subgroups, or, like 12.5K and 19K, are present in the great majority of subgroups (12.5K in A-E; 19K in B-E). A third group of genes, however, appears to be unique for each subgroup. For example, Ad12 (subgenus A) contains unique ORFs with the capacity to encode 29.4K and 30.7K proteins, whereas Ad40 (subgroup F) harbours 19.4K and 30.4K ORFs that seem to be unrelated to other E3 proteins (3,12,13). The location of the unique genes within the E3 regions of subgroups B-E is remarkably conserved. They are positioned either between the 19K and 10.4K, or the 12.5K and 19K ORFs. Between the latter two ORFs the B, D and E subgroups contain homologous ORFs with the capacity to encode 16K and 23K proteins, respectively, while subgenus C has a 6.7K ORF in the equivalent position. Thus far, expression of the corresponding proteins was demonstrated for Ad2, Ad5 (subgroup C) and Ad3 (subgroup B) (3).

Between the 19K and 10.4K ORFs, subgroup B contains 20.1K and 20.5K ORFs, and subgroup E, 27.2K and 29.8K ORFs (3,11,14). The 20.5K ORF was shown to be expressed by Ad3 and Ad7 (15,16). In the equivalent position of Ad19a, a virus classified in subgroup D, we have identified unique 49K and 31K ORFs (10), and (Blusch et al., unpublished). Wold and coworkers proposed 61K and 9K ORFs for Ad9, another virus of subgroup D. This obvious difference in the coding capacity of the two viruses led to the speculation that 49K might be uniquely expressed by Ads causing epidemic keratoconjunctivitis. However, we recently identified the 49K ORF also within the E3 regions of other subgroup D viruses, including Ad9. Moreover, we demonstrated that the 49K protein is synthesized by all subgroup D viruses tested (Blusch et al., unpublished observation). Thus, 49K seems to be a true subgroup D specific protein, although it shares some homology with the 20.1K and 20.5K ORFs of subgroup B (10). Considering the evolutionary relationship between the adenovirus subgenera (see e.g. the phylogenetic trees of E3/14.5K, E3/10.4K, Fig. 4C, D; and reference (17)), it is conceivable that subgroups B, D and E have acquired these unique genes after diverging from subgroups A, F and perhaps C. In any case, it is likely that these unique proteins contribute to the distinct pathogenesis observed for Ads classified in different subgroups (see above). Consistent with this notion, it became recently apparent that all Ads except subgenus B utilize the same primary receptor for infection of cells, the coxsackie-adenovirus receptor (18). Thus, the tropism and the disease pattern can not be accounted for by differential receptor usage, implicating other subgenus specific viral products in these phenomenon.

Interestingly, with the exception of 12.5K and 14.7K, which are primarily found in the cytosol and nucleus, all E3 genes seem to encode transmembrane proteins localized either to the nuclear and Golgi- (11.6K), the endoplasmic reticulum- (ER; 6.7K and 19K) or the plasma membrane (10.4K and 14.5K) (3,19). Although some of the unique E3 proteins, like 11.6K and 6.7K (subgroup C), 16K and 20.5K of subgroup B, and 49K of subgroup D (Windheim and Burgert, in preparation) have been characterized biochemically (3), with the exception of the 11.6K protein, no function has been assigned yet. The 11.6K protein has been proposed to facilitate the release of the virus from the nucleus at the very late stage of infection and, therefore, was recently renamed adenovirus death protein (ADP, (19). Almost all the

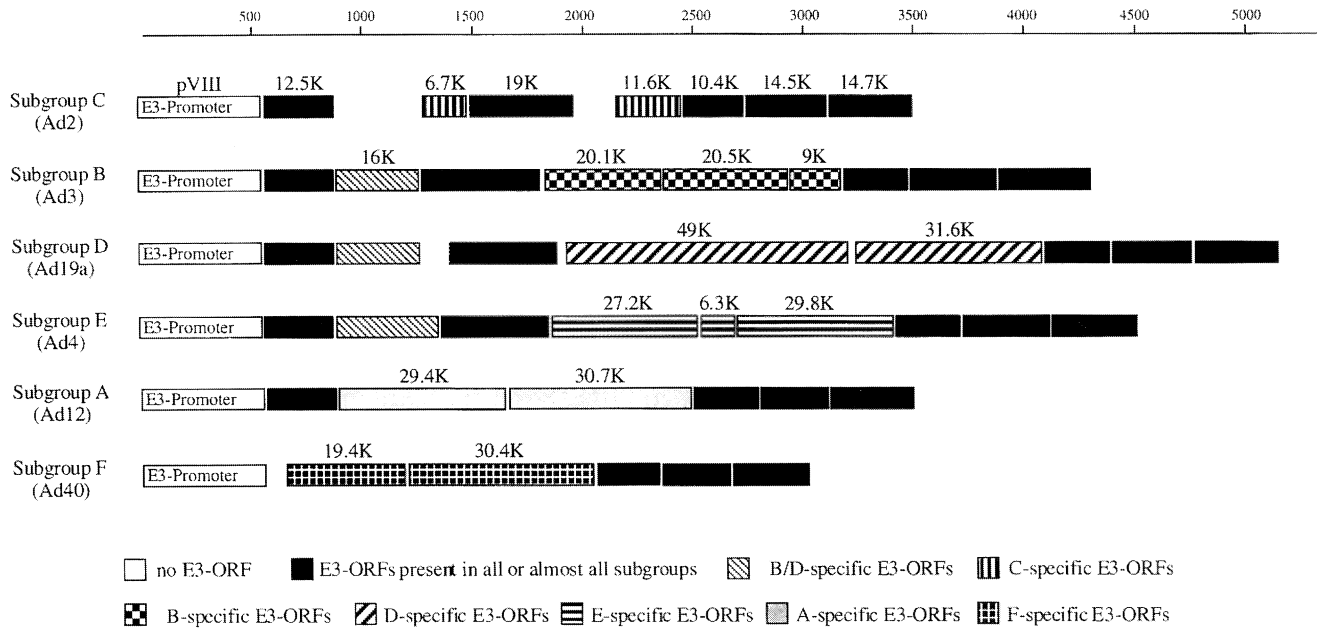

Fig. 1. Organization of the E3 region in the different Ad subgenera. The line on top denotes the size in bp. Open reading frames (ORFs) are indicated as bars and drawn to scale. The shading code is depicted below the figure. Significant homology is indicated by identical shading. Homology to a portion of a protein was neglected. In general, only ORFs greater than 8 kDa are shown (exceptions: 6.7K and 6.3K ORFs). The size of related ORFs is only given once. The figure is based on sequence data for Ad2 (92,93); Ad3 (11); Ad19a (10), this paper and (Blusch et al., manuscript in preparation); Ad12 (12) and Ad40 (13). The sequence of Ad4 has not been released yet, therefore, the arrangement of ORFs in subgroup E was deduced from Fig. 7 in reference (3). Please note that Wold and colleagues have presented a partial map of the Ad9 E3 region (subgenus D) that differs from that we obtained for Ad19a and Ad9 (Blusch et al., manuscript in preparation). For simplicity, the E3 region of only one subtype of each subgroup is shown. Ad11 and Ad35 (subgenus B2) lack the 9K ORF. PVIII is not an E3 protein, but part of its sequence overlaps with the E3 promoter.

functional data reported thus far have been obtained for E3 proteins of Ad2 and Ad5 (subgroup C), therefore, we will limit our discussion to the known immunomodulatory activities described for E3/19K, 10.4K, 14.5K and 14.7K of these viruses (Table 1).

Subversion of Antigen Presentation by the E3/19K Protein

Cytotoxic T cells (CTL) recognize antigenic peptides presented by Major histocompatibility complex (MHC) class I antigens on the cell surface of infected cells. Assembly of MHC class I heavy chain with β_2-microglobulin and peptides occurs in the ER and is assisted by chaperones, such as calnexin and calreticulin (4,20). Peptides bound by MHC molecules are primarily generated by the proteasome in the cytosol and are translocated across the ER membrane by the transporter associated with antigen presentation (TAP) (21). Upon recognition, CTL release perforin and granzymes which promote lysis/apoptosis of the infected cell. Alternatively, CTL may induce apoptosis by interaction of the Fas ligand (FasL) expressed on the T cell surface with the CD95 (Fas/APO-1) receptor on the target cell surface (22).

The E3/19K protein is the most abundant E3 protein expressed by Ads of subgroup C. It binds to MHC class I antigens in the ER and inhibits transport of newly synthesized MHC molecules to the cell surface (23,24). Consequently, T cell recognition *in vitro* is drastically suppressed (25–27). Initially, allogeneic, HLA- or Ad-specific CTL of murine or monkey origin were used to demonstrate this effect, but it has now been confirmed with human Ad specific CTL (4,28). *In vivo* data obtained with animal models strongly support an immunomodulatory role for E3/19K during human Ad infections. Lungs of cotton rats infected with wild type Ad show a less severe immunopathology than those infected with a mutant virus lacking E3/19K (5). Moreover, E3/19K, in combination with the other E3 proteins, can prevent allograft rejection of transplanted islets, and remarkably, suppresses virus-induced diabetes in a murine model (29,30). Integration of E3/19K in Ad-based gene therapy vectors could prolong transgene expression, depending on the mouse strain and the experimental system used (31,32).

With the exception of subgroups A and F, all human adenovirus serotypes (subgroups B-E) express an E3/19K-like protein (Fig. 1) (3,10,33). E3/19K-like proteins share the same basic structure: they are type I transmembrane glycoproteins consisting of 139–153 amino acids (aa, the putative signal sequences are omitted). The luminal part of approximately 106 (+/− 4) amino acids is separated from a 12–15 amino acid long cytoplasmic tail by a transmembrane segment of ~ 23–29 aa (4). Depending on the number of N-linked oligosaccharides the apparent molecular mass of E3/19K molecules varies from 25–35 kDa (10). Although their function, namely to bind to and to inhibit the

Table 1. Immunomodulatory functions of adenovirus E3 proteins

E3 protein	Function	Mechanism
E3/19K	Blocks CTL recognition	Retains MHC class I molecules in the ER
E3/14.7K	Inhibits TNF-α mediated lysis	Inhibits arachidonic acid production by $cPLA_2$
		May block caspase 8 activation
		May block signal transduction from TNFR by binding to FIPs
	Inhibits Fas mediated apoptosis?	May block caspase 8 activation
E3/10.4K–14.5K	Inhibits TNF-α mediated lysis	Prevents activation of $cPLA_2$
	Protects from Fas mediated apoptosis	Down-modulates Fas cell surface expression by enhancing its endocytosis and degradation in lysosomes/endosomes
	Unknown	Down-modulates the EGFR

CTL, cytotoxic T cell; MHC. major histocompatibility complex; TNF-α, tumor necrosis factor α; $cPLA_2$, cytosolic phospholipase A_2; Fas, CD95/APO-1; EGFR, epidermal growth factor receptor, TNFR, TNF receptor; FIP, 14.7K interacting protein.

transport of MHC class I antigens seems to be conserved (10,33), their amino acid sequence homology is surprisingly low (10). Only 26 residues (identity: ~ 18%) are strictly conserved (Fig. 2). Taking into account the conservative amino acid changes the similarity increases to about 30–35% and thus is still remarkably low.

The capacity of E3/19K to bind MHC class I molecules resides primarily in the luminal portion of the protein (34–36) and requires a certain tertiary structure rather than a linear stretch of amino acids. The ability of the Ad2 E3/19K protein to bind to HLA molecules is critically dependent on two intramolecular disulfide bonds formed between Cys11-Cys28 and Cys22-Cys83 (36). The importance of these cysteines for structure and function is reflected by their conservation in all known E3/19K-like proteins (Fig. 2, marked by asterisks). Other conserved amino acids are dispersed throughout the luminal domain but are enriched in a stretch adjacent to the transmembrane segment (10). Alanine scanning mutagenesis of these conserved amino acids led to the identification of amino acids whose substitution by alanine abolishes E3/19K function (Sester et al., in preparation).

The function of the E3/19K protein is based on two activities: The HLA binding activity is combined with the ability to localize to the ER. This latter feature appears to require two structural elements (i) an ER retention signal contained in the transmembrane segment of E3/19K (Sester, Ruzsics and Burgert, manuscript in preparation) and (ii) an ER retrieval signal in the cytoplasmic tail (37–39). The motif for ER retrieval consists of two lysines, positioned either -3 and -4 or -3 and -5 from the carboxy terminus KKXX or KXKXX, where X represents any aa. These dilysin motifs were subsequently also identified in cellular ER proteins. Proteins containing

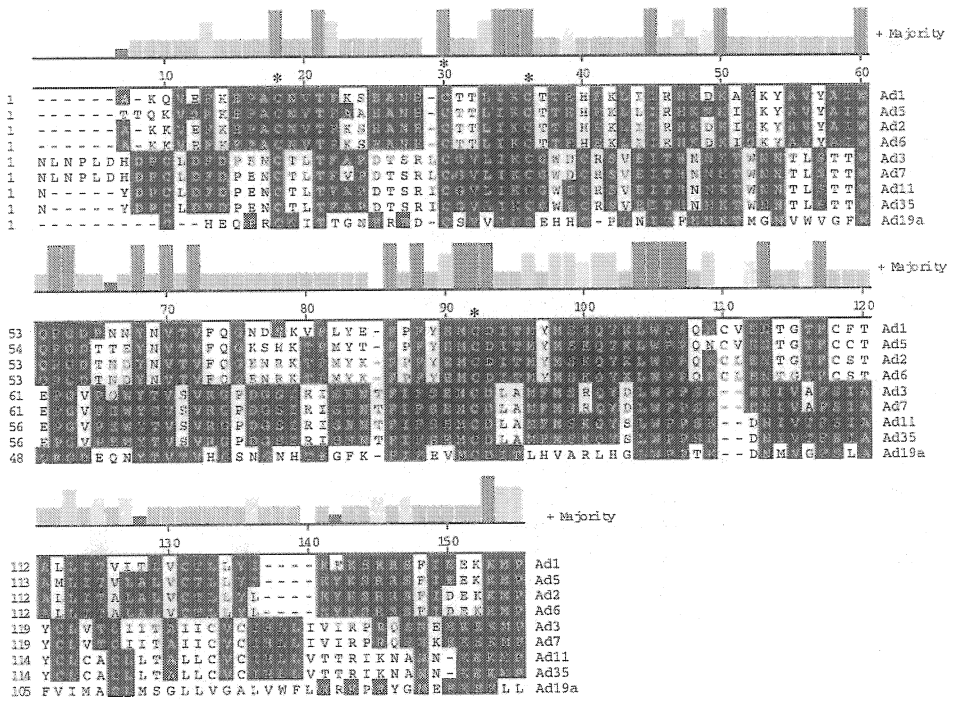

Fig. 2. Amino acid sequence comparison of E3/19K proteins. Alignment was carried out with the DNASTAR software Megalign using Clustal method with PAM250 residue weight table. The putative signal sequences as predicted by the SignalP software (94) are omitted. References to the above sequences are either given in the legend to Fig. 1 or are as follows: Ad1, Y16037; Ad6, G2828254; Ad5 (95), Ad7 (96), Ad35 (14), Ad11 (97). Amino acids that conform to the consensus are shaded. The top bars indicate the degree of similarity to the consensus. The highest bars represent aa identical in all sequences. The asterisks above the sequences denote the 4 Cys which form the proposed disulfide bonds.

these dilysine motifs can reach the cis-Golgi where they are bound by specific cytosolic coat proteins (COPs) which mediate their retrograde transport to the ER (40,41). Mutation of the dilysin motif allows for cell surface expression of E3/19K but the great majority of the protein remains in the ER. Only when the transmembrane segment of E3/19K is replaced by that of a bona fide plasma membrane protein efficient cell surface expression is observed. Thus, the transmembrane segment of E3/19K strongly contributes to ER retention and thereby adds to the efficiency of interaction with MHC molecules (Sester et al., in preparation).

The Ad2 and Ad5 E3/19K molecules, and presumably also the homologous proteins of other adenoviruses, are very promiscuous in that they bind the majority if not all human HLA antigens, albeit with differential affinity (3,42,43). Profound differences exist with regard to the interaction with MHC alleles from other species. Some murine MHC alleles (e.g. K^k, D^d) do not bind E3/19K and hence are not susceptible to its transport inhibition function whereas others (K^d and D^b) strongly associate (44,45). Using hybrid MHC molecules containing domains from E3/19K-binding and non-binding MHC alleles the polymorphic α 1 and α 2 domains of MHC molecules comprising the peptide binding pocket have been identified as being essential for complex formation with E3/19K (44,46). Further characterization of the critical structure by using site directed mutagenesis and antibody binding suggests that the contact site is formed, or is at least influenced, by amino acids within the carboxy-terminal part of the α 2 helix and the amino-terminal part of the α 1 helix (42,46,47). Taking into consideration the broad specificity of E3/19K, the structural element of HLA is believed to be rather conserved. Despite the vicinity to the peptide binding pocket, there is no evidence as yet that E3/19K interferes with peptide binding (39). Additional data suggest that the interaction between E3/19K and MHC molecules occurs soon after translocation of both proteins, probably before or during binding of MHC molecules to calnexin (Sester et al., in preparation). Surprisingly, E3/19K binding does not seem to grossly alter the assembly of MHC class I molecules, rather it abolishes egress of the completely assembled complex out of the endoplasmic reticulum/cis-Golgi compartment. Today, over a decade after the discovery of the E3/19K function (23), it is apparent that interference with antigen presentation is a common strategy of persistent viruses (48,49).

An Alternative Strategy for MHC Repression in Ad12 Transformed Cells

As shown in Fig. 1, Ad12 of the highly oncogenic subgroup A, Ad40 and Ad41 (subgroup F) lack an E3/19K ORF (13) and, therefore, are unable to retain MHC molecules in the ER. Interestingly, in Ad12 transformed cells MHC expression is down-regulated by the Ad12 E1A protein (50), mainly by interfering with MHC transcription (51,52). The molecular mechanism has been extensively studied and seems to involve a differential processing of the transcription factor nuclear factor kappa B (NF-κB) in Ad12 vs. Ad5 transformed cells. However, the data are controversial, for review see reference (53). In addition, other components of the antigen presentation pathway, like TAP transporters and proteasomal subunits, are transcriptionally repressed and this also appears to contribute to the reduced MHC phenotype on the cell surface of Ad12 transformed cells (54). Transcriptional repression extends even to other genes within the MHC complex (53). At present, it is unclear, whether these mechanisms are operating during acute or persistent infections in human. Obviously, the E3/19K function is not required for survival of Ad12, Ad40 and Ad41 in the gut environment where these viruses preferentially replicate, whereas E3/19K might be beneficial during infection of the respiratory tract and other tissues favored by the other adenoviruses.

Inhibition of TNF Mediated Cell Death

Tumor necrosis factor α (TNF) is a pleiotropic cytokine involved in the inflammatory immune response (55). At high concentrations TNF can inhibit the replication of certain viruses (including Ads) *in vitro*, possibly by inducing lysis and/or apoptosis of infected cells. Although it is unclear whether the cytolytic activity represents the major physiologic activity of TNF, several lines of evidence suggest that TNF exerts an antiviral effect *in vivo* (56). TNF acts as a trimer. Two TNF receptors, TNFRI (CD120a or p55) and TNFRII (CD120b or p75) exist, that exhibit a distinct expression pattern (57). Both are members

of the growing TNF receptor/nerve growth factor receptor (TNFR/NGFR) family which includes a number of death receptors, like CD95 (Fas, APO-1), death receptor 3 (DR3), DR4 etc (58). Binding of the cytokine results in clustering of the TNFR and the recruitment to the cytoplasmic tail of several adaptor proteins, such as FADD, TRADD, receptor interacting protein (RIP) and TNFR associated factors (TRAFs). This initiates several signaling pathways culminating in the activation of (i) transcription factors, like NF-κB, AP-1 and c-jun, (ii) cysteine proteases (caspases), which cleave key structural components of cells ultimately leading to apoptosis (59) and (iii) cytosolic phospholipase A_2(cPLA$_2$), an enzyme responsible for production of inflammatory mediators. The signaling pathways involved include the mitogen activated protein (MAP) kinase pathway, ceramide and possibly protein kinase C. While activation of executer caspases, initiated by caspase 8 (FLICE), leads to apoptosis, activation of NF-κB may serve an anti-apoptotic function (60). How these two opposing activities of TNF are orchestrated to give rise to a defined cellular response is currently unclear. Cytolysis by TNF appears to involve the cPLA$_2$ which is activated by MAP kinase and possibly caspase 8 (61) and becomes bound to the plasma membrane where it can release arachidonic acid.

Several years ago it was uncovered that infection of cells with Ad mutants lacking the E1B and/or the E3 region renders these cells susceptible to TNF mediated lysis, whereas cells infected with wild-type Ads are protected (62,63). This suggested that (i) an Ad function exists which induces TNF sensitivity and (ii) E1B and E3 products seem to protect against TNF mediated lysis. Induction of TNF susceptibility was attributed to the multiple activities of the immediate early protein E1A which modulates transcription of cellular genes, induces unscheduled DNA synthesis and leads to deregulation of the cell cycle (64,65). The proteins which contributed to protection from TNF mediated cytolysis were identified as the E1B/19K protein (reviewed in (65) and not further discussed here) and three E3 proteins, the 10.4K, 14.5K and the 14.7K (3). Open reading frames encoding these three proteins are found in all human Ad subtypes examined (Fig. 1).

Function of the 14.7K Product

The E3/14.7K protein varies in size from 122 amino acids in Ad40 to 136 amino acids in Ad3. However, its sequence is markedly conserved between different subgroups (overall homology: 45–59%; 25 aa are identical). This is significantly higher than that observed for the E3/14.5K and E3/19K proteins (overall homology: 26–35% and 28–35%, respectively). Within a particular subgenus, 14.7K proteins are 90% homologous or even identical (Fig. 3). A relatively large proportion of amino acids comprises charged residues confering to the protein hydrophilic properties. 14.7K is localized in the cytosol and the nucleus (66). A structure function analysis of the Ad5 14.7K protein employing in frame deletions and cysteine (Cys) replacement mutations did not reveal a specific subdomain that is required for protection but suggested that functionally critical amino acids are distributed throughout the entire protein (67). Three of the six Cys replacement mutants, with serines 44, 50 and 119 substituted for Cys, were no longer protective against TNF cytolysis. Interestingly, these same three cysteines are strictly conserved while Cys at position 100, 105 and 112, whose exchange had no effect on 14.7K function, are only present in a subset of 14.7K proteins (Fig. 3).

The anti-TNF effect of 14.7K was also demonstrated *in vivo* using heterologous systems (3,68). A recombinant vaccinia virus producing TNF and coexpressing 14.7K (VV14.7TNF) exhibits an increased virulence compared to VVTNF by reversing the attenuating effect of TNF on VV replication (3,69).

Little is known about the molecular mechanism as to how 14.7K interferes with TNF mediated lysis/apoptosis. It has been proposed that 14.7K affects the function of the cytosolic phospholipase A_2(cPLA$_2$), presumably by blocking its translocation to the plasma membrane and thereby the release of arachidonic acid (3). However, synthesis of arachidonic acid is a rather late event upon TNF binding leaving many potential target proteins acting upstream of cPLA$_2$. Using an overexpression system it was recently shown that the 14.7K protein can bind to and inhibit the function of caspase 8 (FLICE) which is recruited to the cytoplasmic tail of the TNFRI and Fas upon binding of the death inducing ligands (9). This activity, although not yet confirmed during natural infection, suggests that 14.7K interferes with signal transduction from the death receptor. As FLICE has been reported to cleave and thereby activate cPLA$_2$, this activity of 14.7K could explain its inhibition of cPLA$_2$. Employing the yeast two

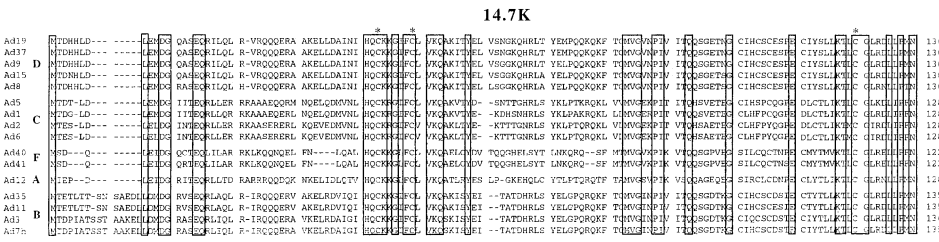

Fig. 3. Multiple alignment of 14.7K amino acids. Alignment of 14.7K protein sequences was carried out with the GeneWorks 2.3 software package (Intelligenetics version, 1994). Residues identical in all serotypes are boxed, identical sequences within D-type adenoviruses are shaded. The asterisks above the sequences denote 3 Cys whose substitution eliminates 14.7K function. Viruses of subgenus D (subgroups are indicated on the left) were obtained from ATCC or were kindly provided by T. Adrian (Hannover, Germany) and G. Wadell (Umea, Sweden). A549 cells were infected with Ad8, strain Freiburg 1127 (100), the prototype viruses Ad9 (ATCC CH38), Ad15 (ATCC Hicks) and Ad37 (ATCC strain G. W.) or Ad19a, a subtype strongly associated with EKC which differs from the Ad19 prototyp (98). Adenovirus DNA was prepared as described (99). The sequence of the E3 region was established by a primer walking strategy. DNA encompassing the entire E3 region was amplified with the primer pair E3consfor/fiberconsrev that bind to conserved regions within the pVIII and fiber gene, respectively. Applied Biosystems TaqFS-Dye-terminator chemistry (Perkin Elmer/Applied Biosystems, Weiterstadt, Germany) was used. Sequences from both strands were analyzed on automatic DNA sequencers ABI 373A or 377 and corrected with the help of the sequence editing program SeqEd (Perkin Elmer/Applied Biosystems, Weiterstadt, Germany). Sequences submitted to Genbank obtained the following accession numbers: Ad8 (Freiburg 1127), AF086567; Ad9, AF086571; Ad15, AF086570; Ad19a, AF086568; Ad37, AF086569. The E3 region sequences of the remaining Ad serotypes were extracted from the NCBI databank (Accession numbers for: Ad1, Y11032; Ad6, Y16037; Ad41, X52198). The sequences of Ad19a and Ad37 are identical. Therefore, the origin of the viral templates was verified by restriction enzyme digestion according to (98).

hybrid system, Horwitz and coworkers identified a number of cellular 14.7K interacting proteins (FIPs). FIP-1 belongs to the family of small GTPases whose function within the TNF signal cascade remains elusive (70). FIP-2 is a protein containing two leucine zipper domains. It has no significant homology to any other known protein. FIP-2 by itself does not cause cell death but it can reverse the protective effect of 14.7K on cell death induced by overexpression of the TNFR intracellular domain or RIP (71). Most remarkably, FIP-3 binds to RIP, a protein recruited to the cytoplasmic domains of TNFRI and Fas (Horwitz, personal communication). RIP has been demonstrated to be crucial for NF-κB activation by TNF (72). Further experiments will be needed to clarify whether these different sets of data reflect multiple activities and multiple target proteins of 14.7K or merely the different systems used.

The E3/10.4–14.5K Proteins also Block lysis by TNF

As mentioned above, two other E3 proteins, E3/10.4 and 14.5K, are able to protect cells from TNF mediated lysis. Like 14.7K, these proteins have been proposed to affect the activity of $cPLA_2$. How this is achieved is completely open. Both proteins are integral membrane proteins which associate non-covalently with each other (3).

The 14.5K product is a type I transmembrane protein consisting of a signal sequence, a short extracellular domain of 20–30 amino acids, a transmembrane region and a cytoplasmic tail of approximately 50 amino acids. The length of the protein seems not to be critical for 14.5K function, since it varies from 107 to 134aa (Fig. 4a). In addition, the sequence homology between the subgroups is with 26–35% significantly lower than that of 10.4K and 14.7K proteins. The Ad5 protein is O-glycosylated and phosphorylated on serines close to the C-terminus (73,74). Another structural feature with potential functional relevance are two conserved motifs in the cytoplasmic tail consisting of the sequence YxxΦ (where Φ represents a bulky hydrophobic aa, e.g. L, I, F). One such motif is found 9 aa from the C-terminus and a second one close to the putative boundary to the lipid bilayer (Fig. 4A). A third YxxΦ motif is identified in 14.5K proteins of subgenus C only (see labeling). YxxΦ motifs are transport signals allowing proteins to be internalized and transported into endosomes or lysosomes (75,76). In addition, the 14.5K proteins of all subgroups contain a proline rich sequence stretch between aa 120–130, with subgroup D, F and A having a second one between aa positions 100–110 and 110–120, respectively. It will be

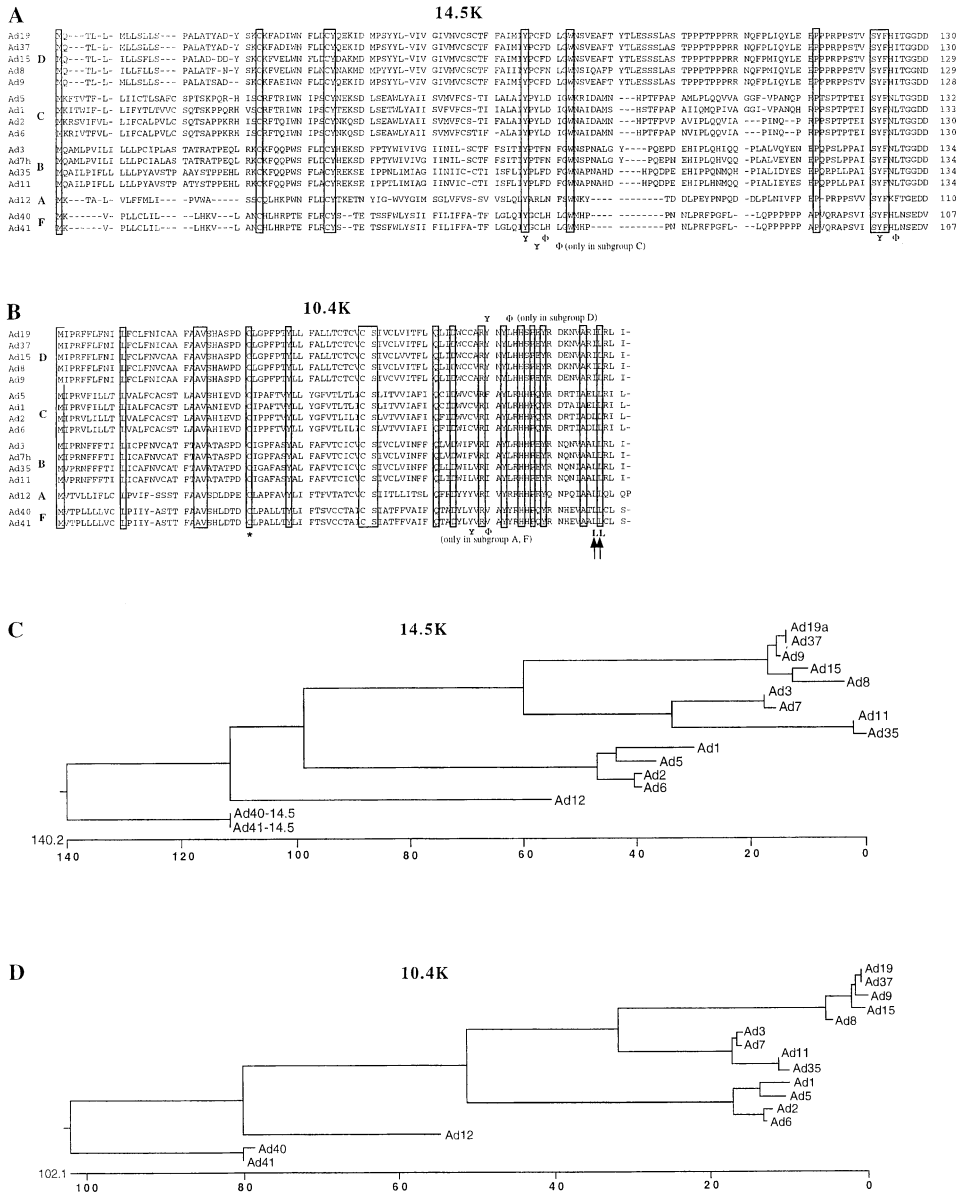

Fig. 4. Amino acid sequence comparison of E3/14.5K (A) and E3/10.4K (B) proteins. The sequences for subgenus D were established as described in the legend to Fig. 3. See also the legends for Figs. 2 and 3 for accession numbers and references of the other sequences shown. Identical residues present in all serotypes are boxed, identical sequences within D-type Ads are shaded. Yxxϕ motifs and the LL motif in 10.4K proteins are indicated. The asterisk marks the conserved cysteine that forms the disulfide bond between the 10.4K species. Dendrograms showing the relationship between 10.4K (C) and 14.5K (D) proteins of different Ad subgenera. Horizontal distances are proportional to the relative sequence deviations between individual aa sequences. The phylogenetic tree was established using the Megaline software (DNASTAR Inc., Version 3.14, clustal with PAM250 weight table). Alignment of 14.5K sequences was further optimized manually.

interesting to determine whether these proline rich sequences are part of a protein interacting domain.

The 10.4K protein exhibits a very hydrophobic character (> 50% of aa are hydrophobic). In contrast to 14.5K, its length of 91aa is, with the exception of the Ad40 and Ad41 versions (90 aa), strictly conserved (Fig. 4b). Furthermore, the sequence homology between the 10.4K proteins of different subgroups is

high (40–52%). Only 18 aa may be exposed on the cell surface while 28 extrude into the cytoplasm. Remarkably, we find again motifs previously shown to mediate transport into endosomes and lysosomes (77). Two leucines (LL), or IL in 10.4K proteins of subgenus D, are present in position -4 and -5 from the C-terminus. The last two aa (IL or LI, in subgroup B-D) may also constitute a dileucine-like motif. Additional YxxΦ motifs can be recognized at the interface between the putative transmembrane segment and the cytosolic portion of the 10.4K proteins of subgroup D and F (Fig. 4B, asterisk). It remains to be demonstrated whether or not these sequences are responsible for targeting of the protein into endocytotic compartments (77), and if so, whether their differential presence and position within the 10.4K proteins of different subtypes reflects distinct intracellular trafficking routes.

10.4K is expressed as two isoforms, in one of which the signal peptide sequence is cleaved while in the other one it remains attached and serves as a second membrane anchor (3). Thus, the latter form is predicted to traverse the lipid bilayer twice. Both 10.4K species are linked by a disulfide bond formed between a cysteine residue at position 31, which is strictly conserved (Fig. 4B). One or both isoforms may form physical complexes with the 14.5K protein and these appear to be expressed on the cell surface (78). This suggests that 10.4K and 14.5K function together (79). However, this issue has not been clarified unequivocally. Apart from the anti-TNF effect, 10.4K and 14.5K down-regulate the epidermal growth factor receptor (EGFR), although a number of studies suggest that only 10.4K is required for this activity (80,81). This discrepancies may be due to the usage of different virus deletion mutants that exhibit altered splicing of E3 mRNAs and, as a consequence, an unpredictably altered expression pattern of E3 proteins. The function of the EGFR modulation for virus reproduction, its potential relationship to the anti-apoptotic activity of the two proteins and for the outcome of an infection *in vivo* is still unknown.

Potentiation of E3 Functions by TNF

The efficacy of E3/19K *in vivo* will depend on whether E3/19K-mediated transport inhibition can be overcome by cytokines like TNF and IFN-γ which stimulate transcription of MHC genes and thereby enhance T cell recognition. Interestingly, TNF is unable to restore MHC class I expression in E3/19K expressing cells *in vitro*. Instead TNF treatment leads to a further reduction of MHC antigens on the cell surface (82). This effect is due to an increased synthesis of E3/19K. Subsequent studies showed that all E3 proteins are upregulated *in vitro* and *in vivo* (83,84), and that this effect is mediated by the cytosolic transcription factor NF-κB which stimulates the E3 promoter (85,86). Moreover, TNF is induced in adenovirus-infected tissue in mice (87). Taken together, Ads devote several proteins to protect infected cells from the cytolytic activity of TNF. In addition, the virus seems to induce TNF in the infected tissue which in turn will upregulate immunomodulatory E3 proteins resulting in the amplification of E3 functions. Hence, this mechanism appears to assure efficient virus reproduction despite the presence of TNF during the early phase of the immune response. Alternatively, it may facilitate persistence in lymphoid tissue.

Inhibition of Fas Mediated Apoptosis by 10.4K–14.5K Proteins

Recently, several groups independently reported that 10.4K–14.5K (also named RID for receptor internalization and degradation) down-regulate the apoptosis receptor CD95 (Fas/APO-1) from the cell surface of Ad infected and E3 transfected cells (6–8). Ad mediated down-regulation of Fas is not due to inhibition of mRNA or protein synthesis but is caused by induction of endocytosis and its subsequent degradation in a lysosomal/endosomal compartment. This was demonstrated by treating infected cells with lysosomotropic agents, such as chloroquine and ammonium chloride, or Bafilomycin A1, an inhibitor of the vesicular ATPase (7,8). Under these conditions degradation of CD95 is prevented and CD95 accumulates primarily in vesicles expressing the lysosomal associated membrane protein 2, presumably the lysosomes. The kinetic of CD95 disappearance from the cell surface of infected cells is significantly more rapid than that observed after inhibition of CD95 transport by Brefeldin A in mock infected cells, indicating an active removal of CD95 from the cell surface rather than intracellular rerouting from the trans-Golgi network (8). The differential sensitivity of the EGFR and Fas to the presence of the 14.5K product suggests that the mechanisms for

down-regulation of these two structurally unrelated molecules may be distinct. Interestingly, cell surface expression of other receptors belonging to the TNFR/NGFR family, like murine TNFR or human CD40, appears not affected by 10.4K–14.5K (6,8).

Removal of Fas from the cell surface by 10.4K–14.5K prevents apoptosis triggered by Fas ligand (FasL) or agonist Fas antibodies. Numerous anti-apoptotic activities of viruses have been described (88,89), that target various steps of the apoptosis cascade. Several viral proteins have homology to cellular Bcl-2 family members, others inhibit the apoptosis mediator p53 while the so called FLIPs interfere with signal transduction from the cell surface receptor (90) and yet others block the caspases themself (59,88). The 10.4K–14.5K activity described above interferes at the earliest time point possible, the interaction of the Fas receptor with its deadly ligand.

At present, it is unclear whether this activity affects Ad-specific CTL or NK cells or an as yet unknown immune response mechanism. However, the presence of this Fas modulating adenovirus function suggests that Fas and FasL are important host effector molecules which the virus counteracts in the early phase of infection. Remarkably, down-regulation of Fas is also observed upon infection of primary cells (8). Thus, this phenomenon is likely to be relevant for the efficient reproduction and, possibly, the persistence of the virus *in vivo*.

Sequence Comparison and Implications

The homology of the E3 proteins within a particular subgroup is generally very high, frequently greater than 90%. In some cases the amino acid sequences are even identical, like the 14.5K sequences of Ad40 and Ad41, the 14.7K sequences of Ad2 and Ad6, the E3/19K sequences of Ad11/Ad35 and Ad2/Ad6. In contrast, E3 protein sequences derived from different subgroups reveal only a poor overall homology ($\sim$ 28 and $\sim$ 32% for 14.5K and 19K proteins, respectively). In E3/19K, 25 of $\sim$ 150 aa, in 10.4K, 17 of 91 aa and 36 of $\sim$ 130 aa in 14.7K proteins are strictly conserved, whereas 14.5K proteins possess only 10 conserved amino acids. The homology is significantly higher among the 14.7K proteins (36 conserved residues) with an overall similarity of $\sim$ 52–59% (using Clustal method with PAM250 weight table). The phylogenetic trees of aligned 14.5K and 10.4K sequences (Fig. 4C and D), and 14.7K (data not shown) correlate with the classification of Ads in the different subgroups. Furthermore, the proposed subdivision of a particular subgenus is also recognized comparing E3 sequences, for example, Ad3 and Ad7 belonging to subgenus B1, and Ad11 and Ad35 to subgenus B2. On the basis of the available E3 sequences, we propose a similar subdivision for subgenus C, Ad1 being more similar to Ad5 and Ad2 to Ad6. Based on the differential hemagglutination of erythrocytes, the largest subgenus D, comprising 31 serotypes, was further divided into DI (e.g. Ad8, 9, 19, 37), DII (e.g. Ad15, 17, 22) and DIII (e.g. Ad28). Interestingly, the dendrograms for the analyzed E3 proteins do not follow this subdivision. Here, the Ad8 sequences have mostly diverged, followed by those of Ad15, Ad9, and then Ad19a and Ad37 whose E3 sequences analyzed so far, turn out to be identical. Sequence identity has previously been observed for the fiber genes of these two viruses, positioned to the right of the E3 region (91). This demonstrates that Ad19a and Ad37 share a larger segment of DNA indicating that Ad37 may originate from a recombination between Ad19a and an unknown Ad.

Conclusions and Perspectives

Human adenoviruses contain a cassette of genes whose products effect primarily host defense mechanisms (Table 1). The E3/19K protein blocks the functional expression of MHC class I molecules and thus CTL recognition, while E3/10.4K-14.5K and 14.7K counteract Fas and TNF induced apoptosis of infected cells by down-regulation of Fas and/or by interfering with signal transduction from Fas and the TNFR, respectively. In combination these measures may extend the lifetime of Ad infected cells, allowing for efficient reproduction of the virus and presumably viral persistence. Elucidation of the precise mechanisms of viral interference with apoptosis should give valuable insight into the physiological regulation of these death receptors (90). Moreover, this knowledge may be therapeutically applied in settings where apoptosis needs to be avoided.

By studying the E3 proteins of subgroup C the functional principals of individual E3 proteins have been elucidated. It will now be important to clarify whether these functions can be extended to the

homologous proteins of Ads classified in other subgroups. The low sequence homology of E3 products encoded by Ads of different subgroups let us assume that they may not function in an identical fashion. Rather, the sequence differences are expected to influence the affinity and the specificity of the interaction with their respective host target molecules.

Apart from the differential activity of the more common E3 proteins the unique E3 products may determine the rather special pattern of disease. By studying these unique proteins we hope to discover novel E3 functions targeting new host molecules.

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Immune Escape by Hepatitis B Viruses

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Abstract. Hepatitis B viruses are DNA viruses characterized by their very small genome size and their unique replication via reverse transcription. The circular genome has been efficiently exploited, thereby limiting genome variation, and leaves no space for genes in addition to those essentially needed during the viral live cycle. Hepatitis B viruses are prototype non-cytopathic viruses causing persistent infection. Human hepatitis B virus (HBV), as well as the closely related animal viruses, most frequently are transmitted vertically from mothers to their offspring. Because infection usually persists for many years, if not lifelong, hepatitis B viruses need efficient mechanisms to hide from the immune response of the host. To escape the immune response, they exploit different strategies. Firstly, they use their structural and non-structural proteins multiplely. One of the purposes is to alter the immune response. Secondly, they replicate by establishing a pool of stable extrachromosomal transcription templates, which allow the virus to react sensitively to changes in its microenvironment by up- or downregulating gene expression. Thirdly, hepatitis B viruses replicate in the liver which is an immunopriviledged site.

Key words: HBV, infection, liver, hepadnaviruses

Introduction

Hepatitis B viruses are non-cytopathic, hepatotropic viruses. Hepatitis B virus infection can cause acute and chronic hepatitis often leading to liver cirrhosis as well as hepatocellular carcinoma. Over 350 million people worldwide are persistently infected with the human hepatitis B virus (HBV) (1). Persistent HBV infection can develop after acute infection in adults, but its main origin is vertical, i.e. perinatal infection of neonates born to HBV infected mothers. Infected individuals who remain HBsAg-positive for more than 20 weeks after primary infection are unlikely to clear the virus and are designated chronic viral carriers (2). While between 2% of infected adults in Europe, USA and Australia to up to 15% in Southeast Asia and Central Africa become chronic carriers of HBV, 95% of infected neonates develop persistent infection (2). It is generally accepted, that HBV infection as such does not result in direct cytopathic effects. Liver damage is a result of the host immune response (3).

Hepatitis B viruses represent prototype viruses for establishing inapparent but productive long-term persistent infection. Like all persistent viruses, they share with their hosts the common objective of survival. After thousands of years of coevolution, both sides have adapted to use rather sophisticated strategies to achieve this goal. From the host's point of view, clearance of the virus will be preferred and the host's immune system will therefore try to destroy the virus. The virus, in contrast, is optimized to coexist with the host to allow sufficient progeny production to infect the next host. In hepatitis B viruses, a well-balanced replication strategy avoids major pathogenic effects and ensures an intimate cross talk between virus and host (4,5).

Virus-Host Interaction in Hepatitis B Virus Infection

Hepatitis B Virus: Particle Structure, Genome Replication and Variation

HBV infected cells secrete different types of particles: besides infectious virions, subviral particles containing no viral DNA are produced in a 10^3- to 10^6-

fold excess (Fig. 1). They consist of lipoproteins containing mainly the HBV small envelope (S) protein. All viral particles have a common antigen on their surface termed hepatitis B surface antigen (HBsAg) which is found in large quantities (1 mg/ml) in the serum of infected individuals. Infectious virions consist of a lipoprotein shell containing three envelope glycoproteins (S, M and L) and a nucleocapsid harbouring the viral DNA with the covalently attached viral polymerase (P) protein (Fig. 2). The structural component of the nucleocapsid is the core (C) protein, which was originally detected serologically as the hepatitis B core antigen (HBcAg). A non-structural, core-related protein is secreted from infected cells and can be detected as HBeAg in patients' sera. Its function as well as the function of an additional, presumably regulatory protein X remains poorly understood. However, X protein is able to

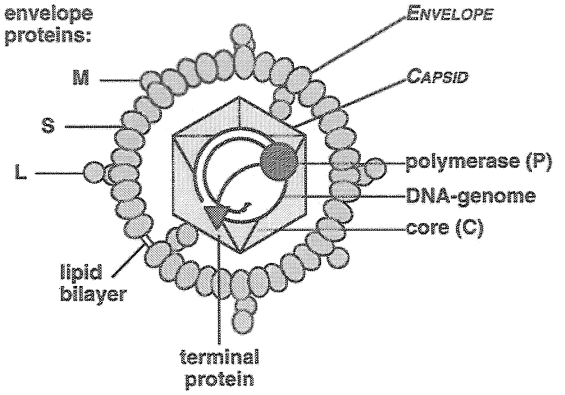

Fig. 2. Structural organization of HBV. The infectious HBV virion consists of a lipoprotein shell containing the three envelope glycoproteins (S, M and L) and a nucleocapsid containing the viral DNA. The capsid consists of 240 subunits of the core (C) protein, and encloses the circular DNA genome and the P protein, which is covalently attached to the negative stand of the viral genome via its terminal protein domain.

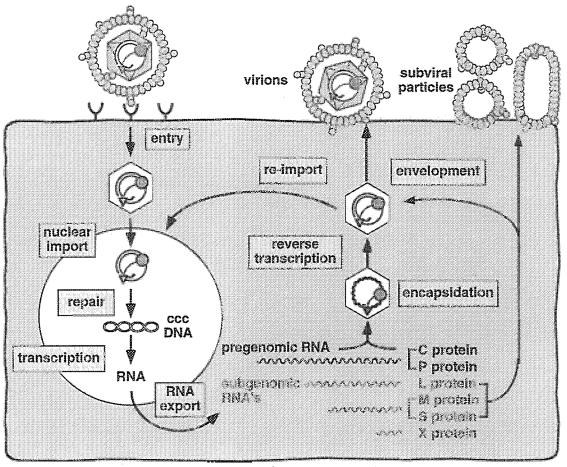

Fig. 1. The basic replication cycle of hepatitis B virus (HBV). After entry into a human hepatocyte, the partially double-streanded DNA genome is imported into the nucleus where it is converted into an episomal, covalently closed circular DNA (cccDNA). cccDNA serves as a template for transcription of a pregenomic and three classes of subgenomic RNAs. The pregenomic RNA serves as a mRNA for the core and polymerase protein; the subgenomic RNAs are translated into envelope proteins L, M and S, and into X protein. Pregenomic RNA and the viral P protein are co-packaged into newly forming capsids where reverse transcription into the viral DNA genome takes place. Progency capsids can either be reimported into the nucleus for amplification of cccDNA or bud into intracellular membranes to acquire their envelope and to be secreted as enveloped virions. In addition to virions, subviral particles containing no viral DNA and consisting mainly of S protein are secreted.

transactivate cellular and viral genes at the level of primary transcription, and is required for the establishment of infection in the woodchuck model of hepatitis B virus infection (6).

HBV is the type member of the family of hepadnaviridae (*hepatotropic DNA* viruses). These small, enveloped DNA viruses replicate their partially double stranded, circular 3,2 kb genome through reverse transcription of an RNA pregenome (for review see: (7,8)). As shown in Fig. 1, the viral genome is delivered to the nucleus and converted into an episomal, unit-length, covalently closed circular DNA (cccDNA). cccDNA serves as a transcription template analogous to the integrated proviral DNA genome in retroviruses. In infected cells, three classes of subgenomic RNAs and a pregenomic RNA can be detected (Figs. 1 & 3). Subgenomic RNAs are translated into the envelope proteins S, M and L and into X protein; the pregenomic RNA serves as mRNA for the core and polymerase protein and is co-packaged with polymerase into newly forming capsids where it is reverse transcribed into DNA. Upon completion of genomic DNA synthesis, progeny capsids can either be directed to the nucleus to establish a pool of cccDNA or bud into intracellular membranes to acquire their glycoprotein envelope and be secreted as enveloped virions (6,7).

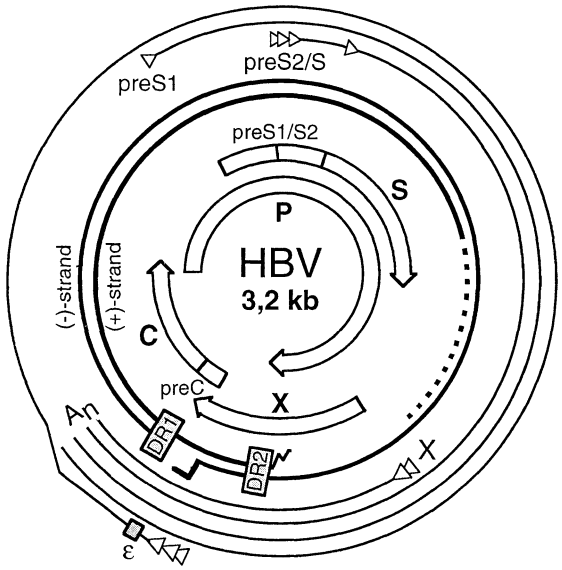

Fig. 3. Genome organization of HBV. The HBV genome as found in infectous virons is a partially double-stranded, circular DNA represented by the bold inner circles. The thin, outer lines represent the different classes of transcripts with the arrowheads as approximate start sites. The open arrows in the center represent the four open reading frames (preC/C, preS1/preS2/S,P, and X).

Mutations of a viral genome occur randomly at frequencies determined by the enzymes the virus uses for replication. As in retroviruses, reverse transcriptase is prone to errors because it lacks proofreading ability. Some of these errors will be propagated or even be selected because of advantages they confer to the mutant virus. However, the extremely efficient organization of the compact hepadnaviral genome and the extreme exploitation of genetic information limits sequence variability. All nucleotides of the 3,2 kb genome have coding function, with more than half having coding function in two or more of the largely overlapping open reading frames (Fig. 3). In addition, regulatory elements, which comprise some 25% of the viral genome, overlap with the coding regions (9). Structural genes have upstream coding regions (preC and preS) that allow to produce different proteins with distinct structure, function and antigenicity from one gene, as exemplified by HBc- and HBeAg. The S-domain is used in all envelope proteins, but modulated in function by aminoterminal addition of extra sequences and further diversified by posttranslational modifications (7). Thus, even single nucleotide exchanges may result in pleiotropic effects. Therefore, most mutations will be lethal for the virus or confer significant disadvantages during viral replication and will represent a major viral population in patients' blood only temporarily. This explains the relatively high sequence conservation among different HBV isolates.

Animal Models for the Study of HBV Infection

Studies on the interaction between HBV and the immune system have been hampered by the fact that HBV infects only humans and chimpanzees. In cell culture, only primary hepatocytes can be infected. Since HBV does not infect cultured cell lines, immonological *in vitro* experiments are limited to the production of antigen after transfection of expression constructs or to the use of synthetic peptides externally loaded onto MHC molecules. Since human blood or tissue samples are limited, systematic studies of HBV infection are rare. Closely related viruses only infect outbred animals, e.g. ducks, woodchucks or ground squirrels, whose immune system is not very well defined. Therefore, HBV transgenic mouse models were developed that have made it possible to gain further insights into the interaction between the virus and the host's immune system (10).

HBV transgenic mice are immunologically tolerant to HBV and its gene products. An HBV specific immune response and acute hepatitis is mimicked by an adoptive transfer of HBV specific cytotoxic T cells into HBV transgenic mice (10). These studies confirmed that HBV-specific cytotoxic T cells bind and kill their target cells *in vivo* by triggering them to undergo apoptosis. However, to a larger extend, T cells contribute to disease severity indirectly by recruiting antigen non-specific effector cells into the liver (11). Interestingly, in HBs transgenic mice, induction of a chronic immune-mediated liver cell injury triggers the development of hepatocellular carcinoma. This suggests that an immune response to HBV is sufficient to cause hepatocellular carcinoma in the absence of viral transactivation, insertional mutagenesis, and genotoxic chemicals (12).

Host Immune Response to HBV Infection

The reasons, why some individuals resolve HBV infection while others do not, remain poorly understood. Variations in host immune responses rather than viral factors are believed to be the critical

variables (3). Upon infection of a new host, HBV has to gain access to the bloodstream. Via the bloodstream, virions reach the liver where they have to pass the sinusoidal lining cells before they get access to their target, the hepatocyte (13). On their way, they have to face the innate immune system of the host including circulating phagocytes, tissue macrophages and, possibly, the complement system. At first exposure to the virus, infection is controlled during the first days by the innate immune system of the host (14). After successful infection, an early, antigen non-specific immune response is induced including activation of natural killer cells (15) and the secretion of cytokines, chemokines and other inflammatory mediators which, besides mediating direct anti-viral activity, recruit additional effector cells. Adaptive HBV-specific immunity is triggered when the virus has eluded the innate immunity and viral replication generates a threshold dose of antigen needed for T and B cell activation.

Antibodies and T cells are the two main antigen-specific effector arms of the immune system to resolve viral infection. Professional antigen presenting cells (i.e. macrophages and dendritic cells) take up viral antigen in the periphery and migrate to lymphoid organs. Naive T cells are primed when they recognize their specific antigen presented on MHC molecules after processing. After clonal expansion, ''armed'' T-cells leave lymphoid organs to exert their effector function. Cytokines present during priming influence the differentiation into Th1 or Th2 cells, which determine whether the adaptive immune response is biased towards a cellular or a humoral immune response (16). Virus-specific CD8 positive T cells are the main effector limb of the antiviral immune response. They are supported in their effector function at the site of infection by T helper cells (for review: (17)). Priming of B cells requires cognate help by specific helper T cells in the lymphoid environment. After clonal expansion, B cells differentiate into plasma cells, which produce antibodies against viral antigens.

Acute hepatitis B virus infection is characterized by a vigorous, polyclonal CD4 and CD8 positive T cell response against HBV. In patients with chronic hepatitis B, on the contrary, a cytotoxic T cell response is barely detectable consistent with the notion that the inability to generate a vigorous T cell response may predispose to persistent infection (3). These data as well as the clinical observation that patients with agammaglobulinemia can clear HBV infection, emphasize the importance of an effective cellular immune response, particularly an HBV-specific cytotoxic T cell response, for the resolution of disease. Antigen specific CD4 and CD8 T cells can be detected in the livers of chronically infected patients (15). It can therefore not be excluded that absence of these T cells from blood is at least in part due to their sequestration to the liver. However, so far only HBsAg, but no HBe- or HBcAg specific CD8 T cells could be isolated at a low frequency from liver biopsies (18).

Besides cytotoxicity, virus-specific T cells contribute to the antiviral immune response by a non-cytopathic mechanism. Adoptive transfer of HBsAg specific T cells into transgenic mice replicating HBV (19) completely abolishes HBV gene expression and replication in all hepatocytes under conditions in which less than 1% of the hepatocytes are destroyed. Viral clearance in this model is cytokine-mediated for it can be completely blocked by antibodies against IFN gamma and TNF alpha (20). Thus, in addition to killing hepatocytes, HBV specific T cells do down-regulate HBV replication via cytokines by a non-cytopathic process in which HBV infected cells participate by production of cellular proteins that interrupt the viral life cycle. In the HBV transgenic mice and in acutely infected chimpanzees, the latter is much more effective than killing of hepatocytes (21,108).

HBV Defence Mechanisms

Silencing of Viral Gene Expression by Cytokines

Very probably, the dual action of cytotoxic T cells described above for HBV transgenic mice also holds true in natural infection: (i) destruction of HBV infected cells and (ii) cytokine-mediated suppression of viral gene expression and replication. Firstly, most immunocompetent adults clear HBV after acute infection with mild or moderate hepatitis. In half of these, disease is even asymptomatic (2). Secondly, studies in animal models of HBV infection show that rapid clearance of the virus occurs in the absence of massive destruction or regeneration of hepatocytes even when all hepatocytes are infected (22,23,108). However, it has so far not been possible to test whether an infection can be cured solely by cytokine-

mediated downregulation of HBV replication as transgenic mice can not be completely cured due to the integrated viral genome (24).

The ability of cytokines to downregulate HBV gene expression and genome replication may serve as a tissue-sparing antiviral strategy by the host. On the other hand, it provides a survival strategy for the virus by leaving nuclear cccDNA as a transcription template untouched (21). In experiments with the animal hepatitis B viruses, it has been impossible to eliminate viral infection without totally eliminating cccDNA (25–27). Thus, downregulation of antigen production while leaving the replication template unaffected may serve as a mechanism of viral persistence.

Interference with the Cytokine Response

A known viral escape mechanism is interfering with cytokine signalling leading to a reduced sensitivity of infected cells to antiviral cytokines. *In vitro* experiments suggest that HBV employs such strategies. It has been shown, that transfection of HBV into interferon alpha sensitive cells resulted in reduced sensitivity to the cytokine (28). The N-terminal fragment of HBV P-protein inhibited the response to interferon alpha and gamma and to double stranded RNA in transfected cells (29). Immunohistochemical studies in HBV infected livers found a negative correlation between expression of interferon-inducible protein beta 2- microglobulin and expression of P protein; this was interpreted as a failure of HBV infected hepatocytes to respond to interferon (30). In addition, HBV core protein has been reported to suppress transcription of the interferon beta gene (31). However, whether and how cytokine signalling is altered during HBV infection remains to be proven in an *in vivo* model.

Tolerance Induction in Vertical Transmission

HBV is one of the blood-born viruses, which can be transmitted to adults, but the main origin of chronic infection worldwide is vertical transmission from mother to child around birth. In children born to HBV positive mothers, persistent HBV infection is believed to be due to neonatal tolerance. Most likely, transplacental infection and/or passage of soluble (HBeAg) or particulate (HBsAg) viral antigens contribute substantially to viral persistence in the infected neonate (3). Transplacental, i.e. intrauterine, infection of the fetal liver has been described (32). Soluble HBeAg is able to cross the placenta and has been demonstrated in the umbilical blood of children of HBeAg-positive mothers. Hence, viral proteins could well be recognized as self-antigens in the thymus of the child and cause clonal deletion of HBV specific T helper cells favoring the development of persistent infection (3). As a model for this, HBeAg-expressing transgenic mice were generated. Non-transgenic progeny of these mice were tolerant to both HBeAg and HBcAg at the T-cell level but produced anti-HBc antibodies (33). These data parallel the immunologic status of neonates born to HBV carrier mothers.

However, neonates born to HBeAg positive mothers can be effectively protected against persistent HBV infection by HBsAg vaccination although their immune system may have also seen HBsAg during its maturation. This means that, comparable to LCMV infection in mice, exposure to virus in utero apparently does not cause a permanent deletion of specific T cells (34).

Replication in the Liver as an Immunoprivileged Site

Viruses causing persistent infection in humans often are found in sites not readily accessible to the host's immune system: e.g. HSV or VZV in neurons, polyomavirus and CMV in the kidney, papilloma virus in skin epithelial cells, EBV in B cells, HIV and HTLV in T cells. Hepatitis B, C and Delta viruses infect the liver.

The liver has a unique architecture. Hepatocytes are separated from the sinusoidal lumen by the space of Dissé and a barrier of liver sinusoidal endothelial cells (LSEC). LSEC have fenestrae with an average diameter of 100 nm and do not form a basement membrane. Although many molecules can diffuse freely through the fenestrae to reach hepatocytes, cells (diameter 7–10 μm) in the sinusoidal lumen do not gain direct access to hepatocytes (35). This is evident in a model of T cell receptor transgenic mice, where the relevant antigen is expressed exclusively on hepatocytes (36). Tumor grafts expressing the same antigen as the hepatocytes are not rejected in these mice unless T cells are activated by inflammatory cytokines (37). Thus, no recruitment of T cells into the liver parenchym and no tissue damage occurs unless two requirements are fulfilled: activation of T cells

and local induction of inflammation in the liver (by bacterial or viral infection or irradiation) (37). This points towards certain prerequisites to induce an immune response in the liver as well as towards induction of specific tolerance.

Hepatitis B virus is a non-cytopathic virus whose replication does not cause cell injury or an inflammatory reaction. Because presentation of viral antigens on hepatocytes obviously does not suffice for recruitment and activation of T cells, the liver seems a suitable site for initial propagation of the virus after infection. Once a virus escaped the host's immune response during the first rounds of replication, HBeAg, subviral particles and virions are secreted by infected hepatocytes. They circulate in the blood and elicit a systemic immune response once reaching a critical antigen concentration. A strong antiviral immune response is triggered that normally leads to hepatitis and elimination of the virus. Nevertheless, in a significant proportion of infected adults the immune response fails to clear the virus from the liver. In HBV infected patients, the cytotoxic T-cell response usually is vigorous and multispecific in acute infection, but weak or undetectable in chronic infection (21).

During chronic infection, the virus appears to take advantage of the liver as an organ able to induce peripheral tolerance. The liver has been known for a long time as a site where immunological tolerance can be induced (38,39) as exemplified by the following observation: oral tolerance, which means antigen specific tolerance to dietary antigens, is lost when a portosystemic shunt prevents portal blood from passing through the liver (40). The mechanisms for tolerance induction in the liver are not well defined. Resident liver cells may be responsible for peripheral tolerance induction as well as the unique microenvironment determined by the continuous exposure to bacteria and endotoxin in the portal blood. To limit immune activation and subsequent liver damage in the physiological situation, immunosuppressive mediators (IL-10, TGF beta, and prostaglandins) are released by sinusoidal lining cells (13). During infection or sepsis, neutralization of these immunosuppressive mediators leads to fulminant liver failure and death (41,42).

Different liver cells (hepatocytes, Kupffer cells and LSEC) seem to contribute to tolerance induction in different ways. Hepatocytes can serve as antigen presenting cells for CD8 positive T cells but lead to premature death of activated T cells (43). Kupffer cells, the resident macrophage population in the liver, are known to effectively induce antigen specific T cell activation *in vitro* (44,45). *In vivo*, they induce tolerance towards phagocytosed antigen (40). LSEC behave like professional antigen presenting cells in that they express MHC II, CD80, CD86 and CD40 and prime naive T cells (44,46). *In vitro*, LSEC modulate T cell responses. Unlike other antigen presenting cells, they do not induce differentiation of naive T helper cells towards a Th1 phenotype and T cells primed by LSEC produce IL-10 (46). In contrast to professional antigen presenting cells, LSEC respond to endotoxin exposure with loss of antigen presenting function (47).

Taken together, the liver is an immunopriviledged site that allows for an effective immune response only after distinct requirements are fulfilled. This might be used by the virus to hide from the immune system during the first rounds of amplification. Furthermore, peripheral tolerance can be induced in the liver at least in part due to its unique microenvironment. HBV may therefore replicate in the liver to evade the immune response during persistent infection.

HBV Infection of Extrahepatic Immunopriviledged Sites

Immunopriviledged sites for a viral infection are tissues or cell types that cannot easily be reached by lymphocytes due to limited access, lack of expression of MHC molecules or minimal expression of viral genes at a level sufficient for survival. In HBV infected humans, Hepatitis B virus nucleic acids were demonstrated in lymph nodes, spleen, gonads, thyroid gland, kidneys, pancreas and adrenal glands (48). Accordingly, HBV replicates in different extrahepatic tissues in HBV transgenic mice (19,49,50). Presumably due to microvascular barriers, HBsAg specific T cells cannot reach HBsAg expressing cells in immunopriviledged extrahepatic sites such as the kidney or the brain in these mice (20,49) whereas systemically applied cytokines can (50).

HBV has been reported to be associated with peripheral blood mononuclear cells (51). Recent data suggest that HBV may be not only taken up but also replicated by mononuclear blood cells at a low level and that these cells may be an extrahepatic viral reservoir (52). Mononuclear cells have also been proposed to serve as source of graft reinfection after

liver transplantation (53,54). Patients who have undergone HBsAg seroconversion may harbor HBV DNA in peripheral blood mononuclear cells for prolonged periods (55). This raises the possibility that extrahepatic reservoirs of HBV might be more difficult to eradicate than infected hepatocytes and may thereby contribute to viral persistence.

On the other hand, persistence of the virus in extrahepatic organs might help to maintain the humoral and cellular immune response and protect patients from reinfection and liver disease. Traces of HBV can be detected in patients' blood for many years after clinical recovery from acute hepatitis. This is possible despite the presence of serum antibodies and HBV-specific cytotoxic and memory T cells which express activation markers indicating recent contact with antigen (56,57). These results suggest that sterilizing immunity to HBV frequently fails to occur after recovery from acute hepatitis. Probably, traces of virus can maintain the T cell response for decades, apparently creating a negative feedback loop that keeps the virus under control.

Selective Suppression of Virus-specific Immunity in the Immunocompetent Host

Besides being presented on MHC class II to CD4 positive T cells, exogenous viral proteins can also be loaded onto MHC class I molecules. Exogenous soluble HBsAg has the remarkable ability to efficiently enter the MHC class I pathway of various cell types where it is processed and presented to CD8 positive T cells (58,59). Antigen-specific B cells e.g. can efficiently deliver HBsAg to the class I pathway and present it to class I-restricted specific cytotoxic T cells which kill the B cells (58). Neutralizing antibodies appear late after infection with non-cytopathic viruses such as HBV, HIV or LCMV. Elimination of neutralizing antibody producing B cells could result in delay in neutralizing antibody production and thus help to establish persistent virus infection (60).

As soluble HBsAg can be presented MHC class I and II restricted, CD4 and CD8 positive T cells are primed and will become effector T cells. To limit extend of an immune response, effector T cells are eliminated dependent on localization, timing and dose of the antigen they encounter. It has been shown that high levels of viral antigen may induce most of the specific antiviral CD8+ cytotoxic T cells at a time and lead to T cell exhaustion (61,62). HBV may exploit this mechanism to persist in an immunocompetent host by producing high levels of HBsAg.

Viral turnover in chronic HBV infection is high with approximately 10^{11} viral particles being produced per day (63). Subviral particles are produced in vast excess (10^3- to 10^6-fold) over virions and are responsible for the enormous quantities of HBsAg found in patients' sera. Thus, at least 10^{14} subviral particles containing HBsAg are produced daily by an infected individual. There are about 10^{12} lymphocytes in the body and precursor frequency is maximally 10^{-4}. This means that there should not be more than 10^8 HBV-specific cytotoxic T cells at any time (24). During acute viral infection, at least a quarter of the specific CD8+ cells can secrete IFN gamma in response to viral peptides (64). However, due to the excess of particulate HBsAg over specific T cells, clonal deletion of the HBsAg-specific T cells after antigen-recognition, stimulation and proliferation is an attractive speculation how tolerance could be induced during acute HBV infection.

Antigen Variation as an Escape Mechanism from Cellular Immune Response

In principle, viruses can escape the surveillance of virus-specific cytotoxic T cells by mutations that alter the relevant T-cell epitope (65). This includes modifying residues that are critical for binding to the MHC molecule as well as recognition by the T-cell receptor. Some of these mutations preserve the normal binding to major histocompatibility complex class I molecules, but present an altered surface to the T-cell receptor. The exact role of these so-called altered peptide ligands *in vivo* is not clear. Altered peptide ligands can either fail to activate, only partially activate or even antagonize T-cells that recognize the original wild-type antigenic peptide (66).

In HBV infection, naturally occurring mutations within T-cell receptor contact sites in immunodominant epitopes of HBV core protein can antagonize cytotoxic T-cell recognition of the corresponding wild-type epitope in selected patients (67). Interestingly, antagonism occurred at low concentrations of the variant peptide. In the patients infected with the HBV variants, antagonist peptides were poorly immunogenic compared to the wildtype peptide suggesting they were unable to elicit an

effective T cell response against the new variant epitope (68). The phenomenon that a cytotoxic T cell response is directed against an initial virus epitope rather than a new variant epitope has recently been defined as ''original antigenic sin'' in analogy to protective antibody crossreactivity (69). Original antigenic sin by T cells leads to impaired clearance of variant viruses infecting the same individual and so may enhance the immune escape of mutant viruses evolving in an individual host. However, immune selection of HBV containing mutant cytotoxic T-cell epitopes seems to be uncommon during chronic infection (70).

Natural Variants of Hepatitis B Virus: HBe-minus Mutants

The most frequently observed HBV variant carries a G-A exchange at nt. 1896 of the viral genome creating a stop-codon in the precore region and abolishing HBeAg synthesis (71,72). Additional mutations abolishing HBeAg synthesis have been described some of which stabilize the ϵ stem loop structure (near the 5′ end of the RNA pregenome) which serves as packaging signal and as origin of genome replication (73,74). This has been discussed to confer an advantage to the mutant virus (75,76). The outgrow of theses variant viruses is accompanied by the disappearance of HBeAg from patients blood and seroconversion to anti-HBe despite ongoing viral replication. Most of the anti-HBe positive patients carry in fact a mixture of wild-type and mutant HBV, at a ratio which varies both from subject to subject and during the course of infection in a given patient (54,77). At present, it is impossible to establish a causal relationship between emergence of HBe-negative HBV variants and the clinical course of disease (78,79).

Accordingly, a significance of the loss of the non-structural HBe protein for viral pathogenicity is not established. Because anti-HBe antibodies do not recognize virions (80), loss of HBeAg can not confer a humoral escape mechanism for the virus. However, it was shown that a membrane-bound form of the HBe protein expressed on the surface of liver cells binds anti-HBe from patient's sera and is probably relevant for an antibody mediated immune response (81).

Because T cell epitopes of e- and core- protein widely overlap, an escape from the cellular immune response by HBeAg negative variants of HBV is unlikely. Probably, the secretion of HBeAg confers an advantage during the establishment of infection that is dispensable during long-term chronic infection. Recent results obtained in transgenic mice expressing HBe- and HBcAg suggest that circulating HBeAg has the potential to preferentially deplete inflammatory HBeAg- and HBcAg-specific Th1 cells which are necessary for viral clearance by apoptosis. This induces a Th2 shift and promotes HBV persistence (82).

Irrespective of their immunological significance, HBe-negative HBV variants could have a growth advantage and therefore be frequently detected in patients' blood. *In vitro* data show that precore expression acts as an inhibitory principle for HBV replication and introduction of a pre-core stop codon mutation strongly enhances the yield of progeny DNA (83). In the transgenic mouse model, overexpression of HBeAg in HBV replicating mice strongly suppressed HBV gene expression and replication (84). A possible growth advantage of the mutant genome may result in its eventual dominance in the virus population present in an infected individual. However, there is no proof for this hypothesis in natural HBV infection, and thus the role of HBe-negativ HBV during the course of hepatitis B virus infection remains unsettled.

Other Natural Variants of Hepatitis B Virus

Although the HBV genome is highly conserved in general due to its compact organization, multiple point-mutations may be found in single viral isolates in patients with chronic HBV infection, and many viral variants may coexist in an infected individual. Mutations affecting all open reading frames in the HBV genome as well as regulatory sequences have been described in patients with acute, fulminant, chronic or even asymptomatic hepatitis B. A link between certain mutations and the outcome or the severity of hepatitis B is discussed but not established so far.

Mutations in the core-gene may be relevant for the natural course of disease and for efficacy of an interferon therapy (for review see: (85,86)). Mutations in the core-promotor region can resemble the phenotype of HBeAg-negative variants and have been associated with high-level viremia or fulminant or severe hepatitis (87–89). In immunosuppressed

patients, variants carrying new binding sites for nuclear factors have been detected (87,90). Mutations or deletions in the preS1- or preS2-region of the HBV genome have been described but their clinical relevance has not yet been clearly defined (91–94). Variants with mutations in the polymerase gene conferring resistance towards antiviral drugs such as Lamivudine (95–97) or Famciclovir (98) are selected during therapy despite a disadvantage in viral replication (99).

Antigenic Variation as Viral Escape from Antibody Recognition

An example of HBV variants with proven clinical relevance are S-gene mutants allowing escape from antibody recognition (100). The main function of a humoral immune response in viral infection is to clear the virus from extracellular compartments in an infected host. In many virus species, there are examples of variants resistant to neutralizing antibodies. The alterations usually affect surface glycoproteins that raise a neutralizing antibody response. In HBV, virus neutralizing antibodies raised against HBsAg confer protective immunity. Recombinant HBsAg produced in yeast is the basis of the currently used vaccine. Furthermore, assays detecting HBsAg in patient's serum are crucial for diagnosis of ongoing infection, and hyperimmunoglobulin preperations containing mainly antibodies against HBsAg are used to prevent infection of a liver graft after liver transplantation. Changes in antigenic epitopes of the surface proteins affect all these applications (for review see: (101)).

Humoral escape mutants of HBV are rare. They were first described in children developing chronic HBV infection in the presence of vaccine-induced anti-HBs antibodies (102). This mutant strain carries a point mutation in the S gene, resulting in an exchange of aa 145 (G145R) in the antigenic, hydrophilic loop of S protein and—due to the multiple use of the S-gene—M and L protein. Further experiments showed that the altered HBsAg fails to bind a range of monoclonal antibodies, binds poorly to polyclonal antisera and is less immunogenic in mice than the wildtype HBsAg (103). This and additional mutants affecting the hydrophilic loop of S were identified around the world, the G145R mutant being the most abundant one (101). By passive immunoprophylaxis with monoclonal anti-HBs antibodies (104) or hepatitis B hyperimmunoglobulins (105–107) to prevent graft reinfection after liver transplantation, HBsAg variants are selected. Thereby, mutants with alterations of aa144 and 145 seem to be associated with a bad clinical outcome after liver transplantation (105).

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MHC Class I–Subversive Gene Functions of Cytomegalovirus and their Regulation by Interferons–an Intricate Balance

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Abstract. Multiple glycoproteins of human cytomegalovirus (HCMV) encoded by the genes *US2, US3, US6* and *US11* interrupt the MHC class I pathway of antigen presentation at distinct checkpoints to avoid recognition of infected cells by cytotoxic CD8+ T lymphocytes. The action of cytokines like interferon (IFN)-γ, IFN-α/β and tumour necrosis factor α (TNF-α) compensate for the viral inhibition and restore antigen presentation in HCMV-infected cells. This finding was explained by their effects on cellular rather than viral genes and reflected by an increase in the production, assembly and maturation of MHC class I molecules resulting in an escape of MHC I from viral control. Here we reproduce the IFN-γ-mediated effect when MHC I-subversive gene functions of HCMV are tested in isolation, but the efficacy of IFN-γ to restore MHC I surface expression in *US2*-, *US6*- and *US11*-transfectants differs significantly. In addition, in HCMV-infected cells IFN-γ strongly affects the synthesis of the *US6*-encoded glycoprotein. Despite the capability of HCMV to block the interferon signaling pathway the IFN-γ driven enhancement of MHC class I and class II expression remains effective provided that cells are exposed to IFN-γ before infection. Our findings illustrate a complex interplay between host immune factors and viral immune evasion functions.

Key words: cytomegalovirus, antigen presentation, MHC, interferon, jak/stat pathway

Introduction

Cytomegaloviruses (CMV) constitute prototypes of the β-subgroup of the family of *Herpesviridae*. CMVs are characterized by their strict species specificity, a protracted replication cycle and their multiplication in a limited number of cell types. Both human (HCMV) and mouse (MCMV) CMVs share large DNA genomes of about 240 kbp encompassing more than 200 separate open reading frames (ORFs) which represent the highest herpesviral coding capacity. A core of genes located in the long sement between approximately 50 to 170 kb of the HCMV genome are closely related between cytomegaloviruses and also conserved in other herpesvirus families (1,2). A hallmark of CMV is the presence of extended virus-specific gene families that are tandemly arranged and cluster as homologous blocks with several members in the flanking regions of the CMV genomes (1,2).

CMVs are subjected to a tight immune control by cytotoxic histocompatibility complex (MHC) class I restricted CD8+ T lymphocytes (CTL) (3,4). CTL monitor the replication of intracellular pathogens such as viruses via a display mechanism mediated by MHC class I molecules (see Fig. 1) which are expressed in virtually all tissues. Peptides derived from viral proteins are presented at the cell surface by MHC class I molecules to CD8+ T cells which either destroy the virus-infected cell by cytotoxicity, secrete cytokines (e.g. IFN-γ), or both. MHC class I molecules are type I transmembrane glycoproteins of about 45 kDa. Noncovalent binding of a soluble 12 kDa light chain, β_2-microglobulin (β_2m) and peptide to the MHC class I heavy chain in the endoplasmic reticulum (ER) results in a stable MHC I complex able to leave the ER for transport to the cell

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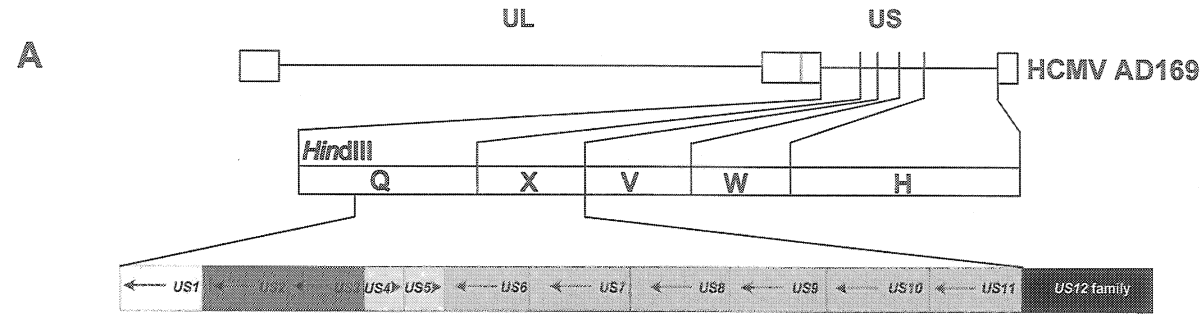

Fig. 1A. Structure of the human cytomegalovirus (HCMV) strain AD169 genome. The unique long (UL) and unique short (US) segments are flanked by reverted repeat sequences as indicated by the terminal boxes. The *US2* and *US6* gene families harbouring the MHC I regulating genes *US2* and *US3*, and *US6* and *US11*, respectively, are highlighted in the context of their neighboring genes *US1, US4, US5* and *US12* which belong to further HCMV gene families. The arrows represent the direction of transcription.

surface along the constitutive secretory pathway of the cell. In the MHC class I pathway of antigen presentation, peptides are generated by proteolytic cleavage in the cytosol. To encounter the peptide binding site of MHC class I molecules, peptides have to be imported into the ER by a specific peptide transporter, transporter associated with antigen processing, TAP, consisting of two subunits, TAP1 and TAP2 which are members of the ATP-binding cassette (ABC) transporter family (reviewed in 5). The transport of peptides by TAP requires two independent but coupled events. In the first step, the peptide is bound to the cytosolic face of TAP, before it is subsequently translocated in an ATP-dependent manner. The formation of trimeric MHC I complexes in the ER is assisted by sequential interactions with molecular chaperones which include calnexin, calreticulin and tapasin (6–8).

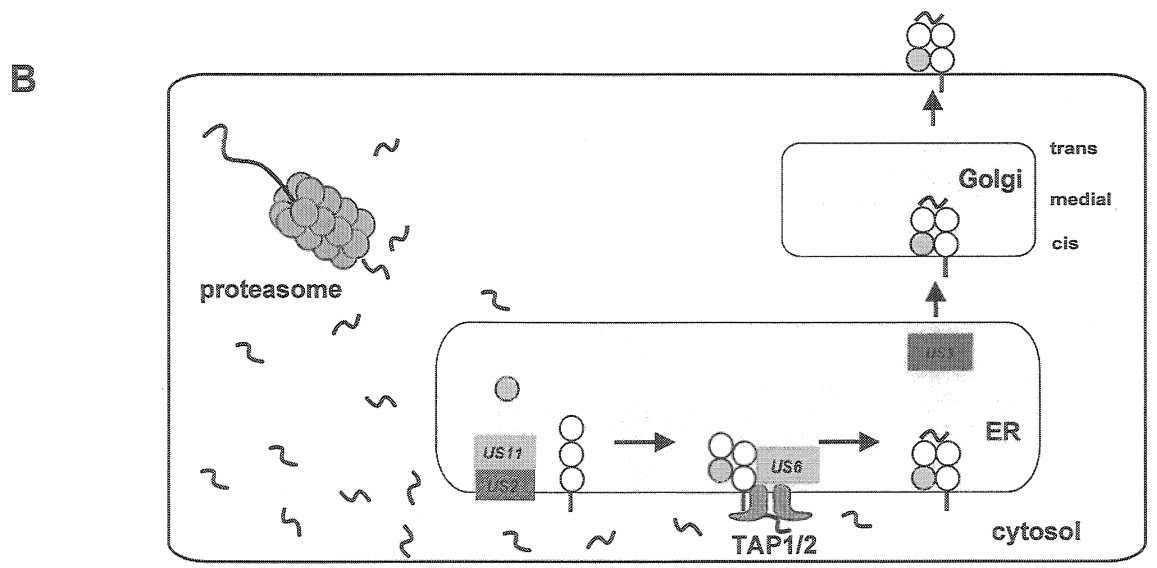

Fig. 1B. The major histocompatibility complex (MHC) class I pathway of antigen processing and presentation. *De novo* synthesized viral proteins or exogenous proteins derived from infecting virions are cleaved by the proteasome to produce peptides. Peptides are translocated across the membrane of the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP1/2). In the ER lumen, peptide bind to MHC class I-β_2-microglobulin heterodimers (unfilled white and filled grey circles) to form ternary complexes. MHC class I complexes exit the ER, pass through the Golgi compartments and reach the cell surface to present the peptide to CD8 + cytotoxic T lymphoctes.

MHC Class I-Subversive Glycoproteins of HCMV are Members of the *US2* and *US6* Gene Families

The selective pressure of CD8 + T cell immunity, the extended time required for replication of the viral genome and the high number of potentially antigenic proteins expressed (> 200) may have led to the evolution of CMV genes that affect MHC class I molecules itself or components of the MHC class I pathway. After infection of fibroblasts both MCMV and HCMV abolish antigen presentation to MHC class I-restricted T cells *in vitro* (9–12). This effect is associated with a downregulation of MHC class I molecules without reducing the mRNA levels for MHC class I gene products (13–16). Although the loss of MHC class I surface expression is observed for both CMVs, the mode of interference with the MHC class I pathway differs thoroughly between HCMV and MCMV and the responsible genes have no homologs between both viruses (17–19).

In HCMV-infected cells MHC class I heavy chains are unstable and steady state levels of assembled MHC class I complexes are strongly decreased (14,15). The analysis of HCMV deletion mutants which had lost the MHC class I reduced phenotype in infected fibroblasts (20,21) guided the identification of four MHC I-subversive open reading frames (orf) within the short segment of the HCMV genome, i.e. *US2, US3, US6* and *US11* (20,22–29; see Fig. 1 and Table 1). The genes are members of two HCMV-specific gene families, *US2* and *US6* (1) coding for small type I transmembrane glycoproteins which are dispensable for virus replication *in vitro* and therefore referred as accessory glycoproteins (20,30). The common phenotype of transfectants expressing *US2, US3, US6* and *US11*, respectively, is the loss of MHC class I molecules on the cell surface, but the molecular mechanisms employed differ. The *US2* family is a clustered pair of two homologous genes, *US2* and *US3*, coding for short-lived glycoproteins of 24 kDa and 32/33 kDa, respectively. Comparing the *US2* and *US3* protein sequence reveals a homology of 23% and a similarity of 56% (25; Fig. 2a). Moreover, their sequences are significantly related to the members of the *US6* gene familiy as shown by the dendrogram depicted in Fig. 3. Therefore, it is tempting to speculate that the members of the *US2* and *US6* gene families have evolved from a common precursor but diverged over time to fulfill different tasks.

Studies of cells stably expressing *US2* provided insights into a novel intracellular pathway used by this HCMV protein to target MHC class I molecules to the cytosol for proteasomal destruction. Cell fractionation experiments demonstrated both a deglycosylated MHC class I heavy chain intermediate and a deglycosylated 20 kDa product of the US2 protein present in the cytosol (23). The physical removal of MHC class I molecules from the ER is ATP-dependent and sensitive to changes in the redox potential of the ER (31). Since both the MHC class I and the US2 intermediate were present in Sec 61p-immune complexes it was suggested that the retrograde transport of MHC I molecules involves the Sec 61p complex, the translocon (23). This is supported by genetic evidence form yeast linking the translocon to a general retrograde transport pathway for misfolded and abnormal proteins in the ER (32). While transcription of the *US2* gene in HCMV-infected cells starts from 3 to 6 h postinfection and is shut off in the late phase of infection (24), the *US3* gene is regulated by multiple copies of an 18-bp repeat present upstream of its promoter (33) resulting in transcription at immediate early times during 1–4 h postinfection which is shut off at early times after infection (25). The US3 protein is immunoprecipi-

Table 1. MHC I-subversive genes of HCMV

Orf*	Gene Family	Size (aa*)	Phase of Expression	Mechanism
US2	*US2*	199	early	SEC61-dependent dislocation of MHC class I heavy chains from the ER into the cytosol for proteolytic destruction
US3	*US2*	186	immediate-early	retention of MHC I complexes in the ER
US6	*US6*	183	early/late	inhibition of peptide translocation by TAP1/2
US11	*US6*	211	early	dislocation of MHC class I heavy chains from the ER into the cytosol for proteolytic destruction

*open reading frame
*amino acid

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A US2     1 MNNLWKAWVGLWTSMGPLIRLPDGITKAGEDALRPWKSTAKHPWFQIEDNRCYIDNGKLFARGSIVGNMSRFVFDPKADYGG
  US3     1 M....KPVLVLAILAVLFLRLADSVPRPLDVVV....SEIRSAHFRVEENQCWFHMGMLYFKGRMSGNFTEKHF...VNVGI

  US2    83 VGENLY..VHADDVEFVPGESLKWNVRNLDVMPIFETLALRLV.....LQGDVIWLRCVPELRVDYTSSAYMWNMQYGMVRK
  US3    72 VSQSYMDRLQVSGEQYHHDERGAYFEWNIGGHPVTHTVDMVDITLSTRWGDPKKYAACVPQVRMDYSSQTINWYLQRSMRDD

  US2   158 SYTHVAWTIVFYSINITLLVLFIVYVTVDCNLSMMWMRFFVC
  US3   154 NWGLLFRTLLVYLFSLVVLVLLTVGVSA.......RLRFI
B US6     1 MDLL...IRLGFLLMCALPT..........PGERSSR.D..........PK....TLLSLSPRQQ...ACVPRTKSHRP.V
  US7     1 MRIQ...LLLVATLVASIVATRVEDMATFRTEKQWQQ.DL..........QYR..REFVKRQLAPK...PKSNIVVSHTV.S
  US8     1 MRRW...LRLLVGLGCCWVTLAHAGNPYEDDDYYYYREDE..........PRQHGEPNYVAPPARQFRFPPLNNVSSYQA.S
  US9     1 MILWSPSTCSFFWHWCLIAVSVLSSRSKESLRLSWSS.DESSASSSSRICPLSN.SKSVRLPQYPRGFGDVSGYRVSSSVSE
  US10    1 MLR......RGS.LRNPLAI..........CLLWWLG.....................VVAAATEE......TREPTYFTCG
  US11    1 MNL....VMLILALWAPVAG......SMPELSLTLF..DE..........PP....PLVETEPLPP.LSDVSEYRVEYSEAR

  US6    50 CYNDTGDCTDADDSWKQLGEDFAH.QCLQAAKKRP.....KTHKSRPNDRNLEGRLTCQRVR..RLLPCDLD.....IHPS.
  US7    63 CVIDGG...NMTSVWRFEGQFNPH.IASEVILHDT.....SGLYNVPHEIQ..NDGQVLTVTVKRSAPADIAKVLISLKP..
  US8    69 CVVKDG...VLDAVWRVQGTFYPEKGIVARVGWSGRRGRKWGRLHAPECLVETTEAVFRLR...QWVPTDLDHLTLHLVPC.
  US9    81 CYVQHG...VLVAAWLVRGNFSDT.APRAYGTWGN..ERSATHFKVGAPQL.ENDGALRYETELPQVDARLSYVMLTVYPC.
  US10   39 CVIQNH...VLKGAVKLYGQFPSP.KTLRASAWLHD.GENHERHRQPILVEGTATATEALY...ILLPTELS.SPEGNRPRN
  US11   57 CVLRSGG..RLEALWTLRGNLSVP.TPTPRVYYQTL.EGYADRVPTPVEDVSESLVAKRYWLRDYRVPQRTKLVLFYFSPC.

  US6   118 ..HRLLTLMNNCVCDGAV.WNAFR..LI...ERHGFFA..VTLYLCCGITLLVVILALLCSITY.......ESTGRG.....
  US7   132 ..VQLSSGQYECRPQLQLPWVPRPSSFMYDSYRLWYEKRWLTIILYVFMWTYLVTLLQYCIVRFIG.TRLFYFLQRN.ITIR
  US8   144 ..TKCKP..MWCQPRYHIRYFSYGNSVD.NLRRLHYEYRHLELGVVIAICMAMVLLLGYVLARTVYRVSSAYYLRWH....A
  US9   155 ..SACNRSVLHCRPASRLPWLPLRVTPS.DLERLFAERRYLTFLYVVLVQFVKHVALFSFGVQVACCVYLRWIRPWVRGRHR
  US10  113 YSATLTLASRDCYERFVCPVYDSGTPMG.VLMNLTYL..WYLGDYGAILKIYFGLFCGACVITR....SLLLICGYY.....
  US11  134 ..HQCQTYYVECEPRCLVPWVPLWSSLE.DIERLLFEDRRLMAYYALTIKSAQYTLMMVAVIQV...FWGLYVKGWL...HR

  US6   178 .IRRCGS.........
  US7   210 FTGKPTYNLLTYPVKG
  US8   217 CVPQKCEKSLC.....
  US9   234 ATGRTSREEEAKDD..
  US10  182 .PPRE...........
  US11  207 HFPWMFSDQW......
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Fig. 2. Amino acid sequence alignment of the *US2* family members *US2* and *US3* (**A**) and the *US6* family members *US6* through *US11* (**B**). Numbers indicate the amino acid positions within the published amino acid sequences (1). Amino acids identical in more than 50% of the genes are highlighted. Points indicate artifical gaps introduced to achieve maximal amino acid matching.

tated with β_2m-associated peptide-loaded MHC class I molecules. In contrast to *US2*-expressing cells, *US3*-transfectants do not show a rapid degradation of MHC class I molecules but accumulate stable MHC class I complexes in the ER and prevent their transport to the cell surface (25,26). Since the expression of the *US3* gene is activated by cellular factors and independent of viral protein synthesis, one may speculate that the US3 glycoprotein is also able to limit presentation of viral peptides in cells nonproductively infected with HCMV.

Two of the six members of the *US6* gene family also interrupt the MHC class I pathway of antigen presentation, i.e. *US6* and *US11*. Another family member, the *US9*-encoded glycoprotein was shown to be implicated in the cell-to-cell spread of HCMV in polarized epithelial cells (34), indicating that the accessory glycoproteins of the *US6* family have diverse biological functions. While the overall sequence homology between the US6 polypeptides is in the range of about 25% and includes also *US2* and *US3* (Fig. 2 and 3), the *US6* family members are characterized by two areas of sequence homology (1). The core motif of the first region is defined as C(VY)X(DQKR)(7–10)WXXXGXF where the bracketed residues are alternatives and X stands for

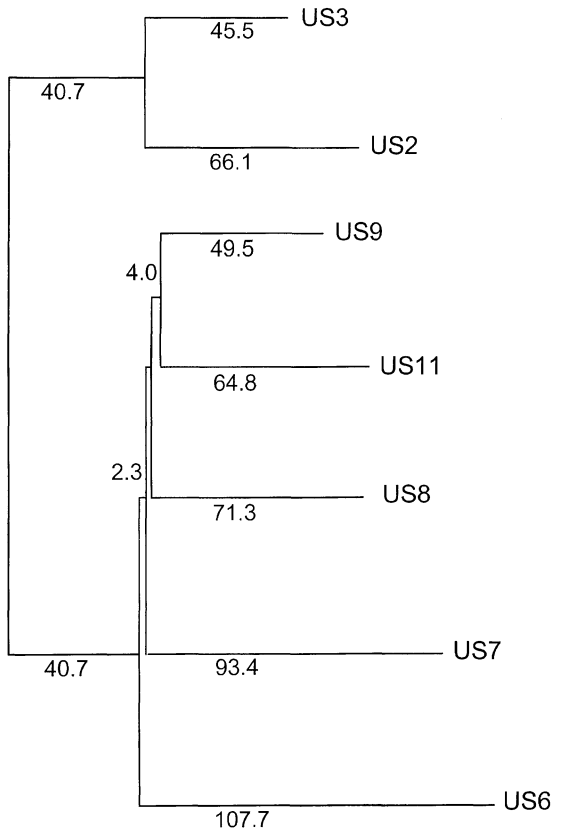

Fig. 3. Dendrogram showing the amino acid relatedness of the *US2* and *US6* gene families. The dendrogram was based on a multiple alignment of the complete amino acid sequences of the *US2* and *US6* gene family members using the CLUSTAL software (PC/GENE release 6.85, Intelligenetics Inc., CA). Horizontal distances are proportional to the relative sequence deviations between individual amino acid sequences and indicated as arbitiary values.

any residue. The motif of the second region is defined by cystein and proline residues: PCXXC(4–6)CXPWXP (1).

Phenotypically, the *US11*-encoded 33 kDa glycoprotein acts upon MHC class I molecules like US2 and dislocates nascent MHC class I molecules from the ER back to the cytosol where they are rapidly degraded (22). Remarkably, the expression kinetics of *US11* parallels that of *US2* (24,35), but their preference for MHC class I alleles differs as deduced from the fact that the US2 and US11 proteins exhibit a different ability to attack allelic forms of murine MHC class I heavy chains (36). On the other hand, both US2 and US11 leave out HLA-C and HLA-G histocompatibility antigens which escape from degradation (37). This might be due to the fact that natural killer (NK) cells are blocked by HLA-C and HLA-G alleles.

Unlike the US2, US3 and US11 proteins the *US6*-encoded 21 kDa glycoprotein (gpUS6) does not directly interact with MHC class I but shuts off the TAP1/2-mediated peptide transport into the ER (27–29). gpUS6 does not affect peptide binding to TAP1/2 but prevents the translocation step of the peptide ligand across the ER membrane. The US6 protein is found associated with the recently identified assembly complex consisting of TAP1, TAP2, MHC class I-β_2m, calreticulin and tapasin, and it binds also to calnexin (27). The inhibiton of peptide transport is accomplished despite the significantly augmented expression of TAP1 and TAP2 molecules in HCMV-infected fibroblasts (21). The expression kinetics of the US6 protein during permissive infection starts in the early phase and correlates with the inhibition of peptide transport. Detailed analysis of US6 transcripts revealed that transcription is driven from different initiation sites at early and late times postinfection, respectively (35). US6 synthesis reaches peak levels not before the late phase of infection when *US3*, *US2* and *US11* gene expression becomes almost silent (27).

Restoration of MHC I Functions by Cytokines

Complete escape from immune control would result in the uncontrolled replication of the virus. This would harm and finally kill the host and thus cease the dissemination of the virus. The efficacy of virus-specific CTL which can control CMV replication *in vivo* (3,4) indicated that the viral immune evasion mechanisms operate *in vivo* with a limited degree of effectiveness and suggested further that the antigen presentation function of CMV-infected cells is a matter of regulation. *In vitro* data provided evidence that certain cytokines, i.e. interferon γ (IFN-γ), type I interferons (IFN-α and IFN-β) as well as tumor necrosis factor-α (TNF-α) are able to restore antigen presentation and CTL recognition of fibroblasts infected with MCMV and HCMV, respectively (12,38). The cytokines compensate the MHC I inhibition by both viruses despite the fact that the mechanisms that are operative clearly differ. Among these cytokines, IFN-γ is most efficient in restoring antigen presentation of CMV-infected fibroblasts, but

type I interferons as well as TNF-α have also a significant effect (12). Two explanations of the effects on antigen presentation in CMV-infected cells are possible. First, all of these cytokines have been shown to exert strong inhibitory effects on CMV replication by inhibiting expression of late genes and nucleocapsid assembly (39), raising the possibility that the expression of MHC I-subversive genes can be suppressed by interferons. Alternatively, the effect could be explained by the fact that the factors influence cellular genes, i.e. stimulate MHC class I and β_2m gene expression (40–42). The potency of IFN-γ could be due to its ability to stimulate transcription of further genes, e.g. TAP1, TAP2, tapasin and MHC-encoded subunits of the proteasome (43) which might increase the generation and supply of viral peptides for MHC I assembly.

To address the first possibility, we tested modulation of MHC I expression by IFN-γ in stable transfectants expressing the HCMV-subversive genes *US2*, *US6* and *US11* in isolation. Fig. 4a and Fig. 4b show that *US2*-, *US6*- and *US11*-transfectants display a drastically reduced MHC class I surface density compared to untransfected control cells. Exposure of cells to graded concentrations of IFN-γ increases MHC class I expression in untransfected control cells in a dose-dependent order. The IFN-γ effect is reproduced in the presence of MHC I-subversive HCMV gene functions, albeit to an extent depending on the *US* gene expressed (Fig. 4A,B). After stimulation with IFN-γ, a surplus of MHC I molecules escapes from the control by the viral inhibitors and reaches the cell surface, where few MHC I molecules suffice for CTL recognition.

Next, we investigated whether IFN-γ displays effects on viral genes responsible for MHC class I downregulation. Pre-incubation of fibroblasts with IFN-γ increases the assembly of MHC class I complexes in cells infected with HCMV for 72 h dose-dependently reaching higher levels than mock-infected controls (12; Fig. 5a). At this time the *US6* gene is most abundantly expressed in HCMV-infected fibroblasts (27). We therefore tested whether the gpUS6-mediated inactivation of TAP1/2 is manifest under these conditions. Peptide translocation by TAP1/2 was found almost efficient as in mock-infected controls (F. Momburg and H. Hengel, data not shown). This is consistent with our finding that IFN-γ treatment strongly impairs gpUS6 synthesis in HCMV-infected cells (Fig. 5b). It will be interesting

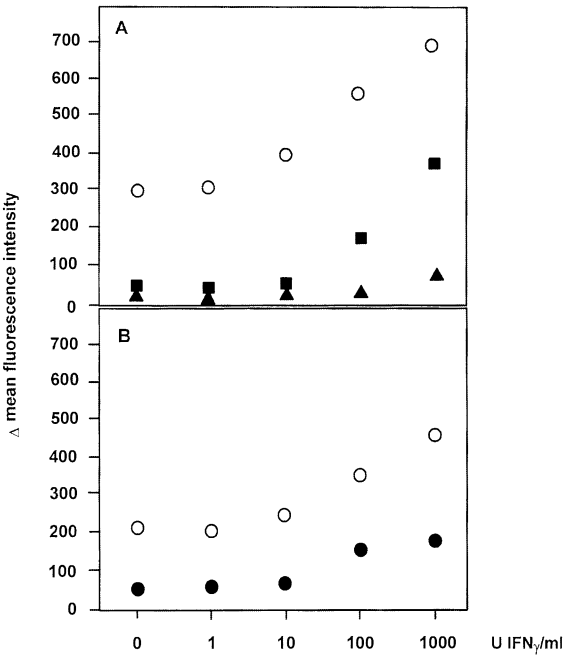

Fig. 4. Interferon-γ (IFN-γ)-mediated restoration of MHC class I surface expression in *US2* and *US11*-transfected LC-5 cells (**A**) and *US6*-transfected HeLa cells (**B**). Cytofluorometric analysis of MHC class I surface expression of cells transfected with pcDNAI-US2 (filled triangles), pcDNAI-US11 (filled rectangle) and pcDNAI-US6 (filled circles), respectively, and untransfected HeLa and LC-5 control cells (open symbols). Cells were incubated with graded doses of IFN-γ for 48 h before stained with MAb W6/32 recognizing human MHC class I molecules followed by FITC-labeled goat anti-mouse IgG antibodies. The data are given as mean fluorescence intensity values of W6/32-labeled cells minus control staining with the second antibody only.

to learn which of the *US6* transcription units are sensitive to IFN-γ and whether the expression of *US2*, *US3* and *US11* are also sensitive to IFN-γ, since transcription of these genes is under different control.

Remarkably, restoration of antigen presentation of fibroblasts strictly requires pretreatment of cells with cytokines before CMV infection, while IFN-γ had no effect on already infected cells (12,38). Likewise, the inhibition of CMV replication by IFN-γ critically depends on pre-exposure of cells before infection (39). These observations predicted recent reports demonstrating that CMVs interfere with the host cell response to IFNs (44,45).

Several findings from *in vivo* studies relate to the effects of cytokines on antigen presentation. First, the antiviral effector function of adoptively transferred $CD8^+$ CTL into MCMV-infected mice requires INF-γ

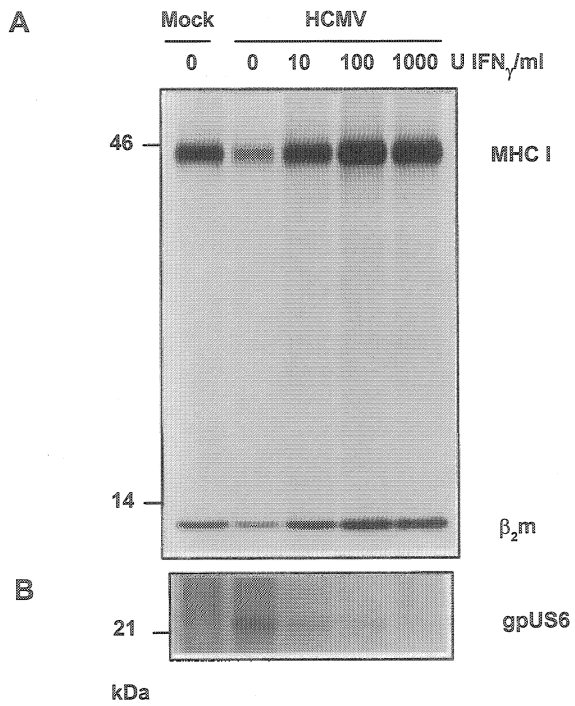

Fig. 5. Interferon-γ (IFN-γ)-pretreatment of fibroblasts before HCMV infection restores MHC class I assembly (**A**) and inhibits synthesis of the US6 glycoprotein (gpUS6) (**B**) in a dose-dependent fashion. Human foreskin fibroblasts were exposed to IFN-γ as indicated for 48 h before infected with HCMV AD169 (multiplicity of infection (moi) = 5) for 72 h. Cells were metabolically labeled with [^{35}S] methionine for 90 min and lysed in 1% NP 40 lysis buffer. All lysates used for immunoprecipitation were adjusted to ensure comparability in quantitative terms. MHC class I-β_2-microglobulin complexes were immunoprecipitated using MAb W6/32 and protein A sepharose, gpUS6 molecules were retrieved using a polyclonal rabbit antiserum raised against an US6 peptide. Immune complexes were eluted with sample buffer and analyzed by 11.5%–13.5% PAGE. Gels were dried and exposed to films for two days.

(38), compatible with the notion that this cytokine regulates antigen presentation of infected cells *in vivo*. In addition, the extraction of antigenic viral peptides from MCMV-infected organs demonstrated direct evidence for a pivotal role of IFN-γ *in vivo*. Efficient generation of antigenic peptides from viral proteins and the subsequent loading onto MHC class I molecules could be decreased by neutralization of INF-γ and restored in immunocompromised mice by INF-γ administration (46). The observation that IFN-γ is able to restore antigen presentation of adenovirus- and herpes simplex virus-infected cells which also subvert immunity by MHC I-reactive proteins (47,48) points to a more general role of IFN-γ to promote antiviral CD 8+ T cell effector functions against persisting viruses.

HCMV Interference with the Jak/Stat Pathway

MHC class II genes are constitutively expressed only in few cell types, i.e. B lymphocytes, dendritic cells and thymic epithelial cells. In MHC class II negative cells, IFN-γ is the most potent inducer of MHC class II transcription. IFNγ stimulates MHC class II gene expression by activating the Jak/Stat signal transduction pathway (49,50). In this pathway a cascade of events is inititated after IFN-γ binding to its receptor. This receptor is associated with the Janus kinases (Jaks) Jak1 and Jak2, both of which become phosphorylated upon IFN-γ binding, as well as the cytoplasmic tail of the IFN-γ receptor itself. Each phosphorylated IFN-γ-receptor chain forms a binding site for a member of the familiy of signal transducers and activators of transcription (Stats), Stat1α. After docking at the receptor, Stat1α is phosphorylated by the Jaks and migrates to the nucleus where it binds to specific sites present in promotors of IFN-γ-inducible genes. Both HCMV and MCMV disrupt the IFN-γ-mediated induction of MHC class II transcription through the Jak/Stat pathway and thus antigen presentation to CD4+ T cells (44,45; Fig. 6, lowest panel). Despite the common phenotype, the underlying viral mechanisms appear different. In contrast to HCMV, MCMV infection interferes with the induction of MHC class II genes at a stage downstream of Stat1α activation and nuclear translocation (45). In HCMV-infected cells levels of Jak1 are significantly decreased, obviously due to an HCMV-associated enhancement of Jak1 protein degradation (44). Since signal transduction by type I interferons is also Jak1-dependent, it is readily clear that HCMV interferes by this means also with IFN-α and IFN-β mediated responses (51). As found for MHC class I-restricted antigen presentation (12,38), preincubation of fibroblasts with IFNγ preserves induction of MHC class II gene expression in HCMV-infected fibroblasts with an efficiency almost comparable to mock-infected IFNγ-stimulated contol cells (Fig. 6). From this result one may speculate whether the HCMV genes which interfere with the IFN-γ driven induction of MHC

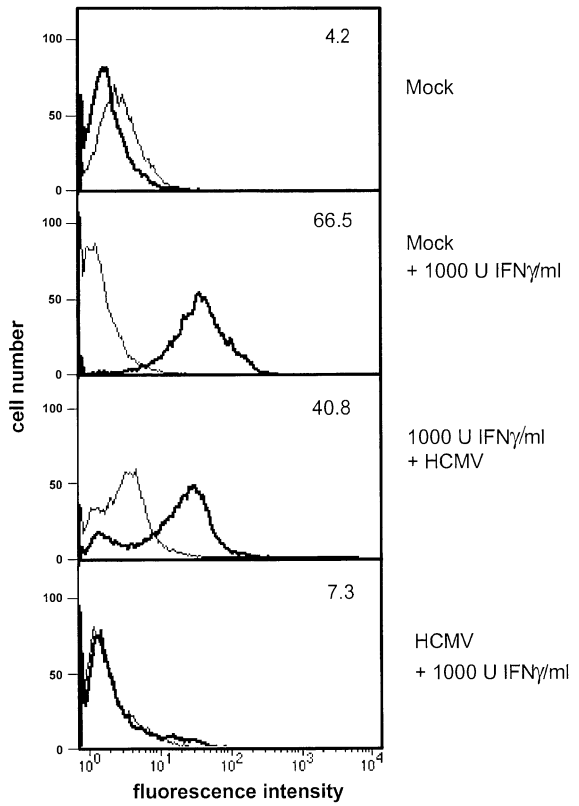

Fig. 6. Induction of MHC II molecules in HCMV-infected fibroblasts by preincubation with Interferon-γ (IFN-γ) before infection. Human foreskin fibroblasts were either mock treated (top panel) or exposed to 1000 U IFN-γ/ml for 96 h (second top panel) or exposed to 1000 U IFN-γ/ml for 24 h before infection with HCMV AD169 (multiplicity of infection (moi) = 5) for additional 72 h (second lower panel) or infected first with HCMV AD169 (moi = 5) before exposed to 1000 U IFN-γ/ml for 72 h. Cells were stained with MAb 2.06 recognizing human MHC class II molecules followed by FITC-labeled goat anti-mouse IgG antibodies (bold line) or with second antibody only (narrow line) and analyzed by cytofluometry. Mean fluorescence intensity values of MAb 2.06-labeled cells minus control staining with the second antibody only are given in each histogram.

class II transcription might be counterregulated by IFN-γ itself.

Conclusions and Perspective

The complete course of permissive HCMV infection is covered by the expression of MHC class I-subversive glycoproteins. They represent a paradigm for 'natural' immune modulators which have been highly adapted to their functions during the coevolution of CMVs with their hosts over millions of years. The viral inhibitors have proven to be valuable tools for the elucidation of molecular mechanisms in the MHC class I pathway of antigen presentation. The bewildering array of MHC class I-subversive genes in cytomegaloviral genomes may reflect the urgent need of these viruses to keep pace with the evolution of MHC class I genes as well as antagonistic effects mediated by cytokines. The intricate balance between host immune control and viral evasion ensures both the host's freedom from harmful disease manifestations and the need of CMVs to replicate sufficiently and to spread. The identification of the genetic basis for the subversion of the IFN response and MHC class II functions is a goal of prime importance for future research. It will be of interest to see whether one or multiple genes were used to prevent MHC class II expression. The number of cytomegaloviral genes affecting immune and cellular functions that have been identified to date probably represents just the tip of an iceberg. CMV genomes are promising sources for novel regulators for immune and nonimmune functions. Our knowledge about viral modulators has implications for the understanding of CMV biology, for the prevention of disease manifestations in patients at risk and for vaccine development.

Acknowledgments

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PART B:

Acquisition of Cellular and Viral Genes by Herpes and Iridoviruses

Virus Genes 21:1/2, 51–64, 2000

Marek's Disease Herpesvirus Transforming Protein MEQ: a c-Jun Analogue with an Alternative Life Style

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Abstract. In order to adapt to and to cope with an often hostile host environment, many viruses have evolved to encode products that are homologous to cellular proteins. These proteins exploit the existing host machinery and allow viruses to readily integrate into the host functional network. As a result, viruses are able to maneuver their journey seemingly effortlessly inside the host cell to achieve ultimate survival. Such molecular mimicries sometime go overboard, allowing viruses to overtake the cellular pathways or evade the immune system as do many of the retroviral oncogenes. Retroviral oncogenes are derived directly from host genes, and they are virtually identical to host genes in sequences except those mutations that make them unregulatable by host. Oncogenic herpesviruses also encode oncogenes, or transforming genes, which have independently evolved and are distantly related to host genes. However, these genes do share consensus structural motifs with cellular genes involved in cell growth and apoptosis and are functional analogues to host genes. The Marek's disease virus oncoprotein, MEQ, is one such example. MEQ is a basic region-leucine zipper (bZIP) transactivator which shares extensive homology with the Jun/Fos family of transcription factors within the bZIP domain, but not in other regions. Like all other bZIP proteins, MEQ is capable of dimerizing with itself and with a variety of bZIP partners including c-Jun, B-Jun, c-Fos, CREB, ATF-1, ATF-2, and SNF. MEQ-Jun heterodimers bind to a TRE/CRE-like sequence in the *meq* promoter region and have been shown to up-regulate MEQ expression in both chicken embryo fibroblasts and F9 cells. In addition, the bZIP and transactivation domains are interchangeable between MEQ and c-Jun in terms of transforming potential; i.e. MEQ can functionally substitute for c-Jun. These properties enable MEQ to engage in host cell processes by disguising itself as c-Jun. On the other hand, there are properties of MEQ notably different from c-Jun, which include its capability to bind RNA, to bind a CACAC-bent DNA structure as a homodimer, to inhibit apoptosis, and to interact with CDK2. MEQ's subcellular localization in the nucleolus and coiled body, is also different from Jun/Fos family of transactivators. These unique features may provide the MEQ with additional facility in regulating MDV replication, establishing latency, and cellular transformation. In this review, we will attempt to summarize the past research progress on MDV *meq*, with a focused on the similarities and differences between MEQ and cellular proteins, and between MEQ and other viral oncoproteins.

Key words: apoptosis, bZIP, coiled body, herpesvirus, Jun, nucleolus, oncogene, transactivation

Introduction

Viruses come in different sizes, shapes, structures, and genomic organizations. Irrespective of their divergence and complexity, one common theme for all viruses is to survive and to produce progeny in host. Different viruses have evolved different means to achieve the ultimate survival in an often hostile host environment. One strategy commonly used by oncogenic viruses is to encode products mimicking cellular proteins involved in growth, differentiation and apoptosis, as well as those in immune surveillance. The viral proteins by virtue of their similarity to the host proteins integrate readily into, and sometime overtake the existing cellular pathways controlling signal transduction, cell cycle progression, DNA

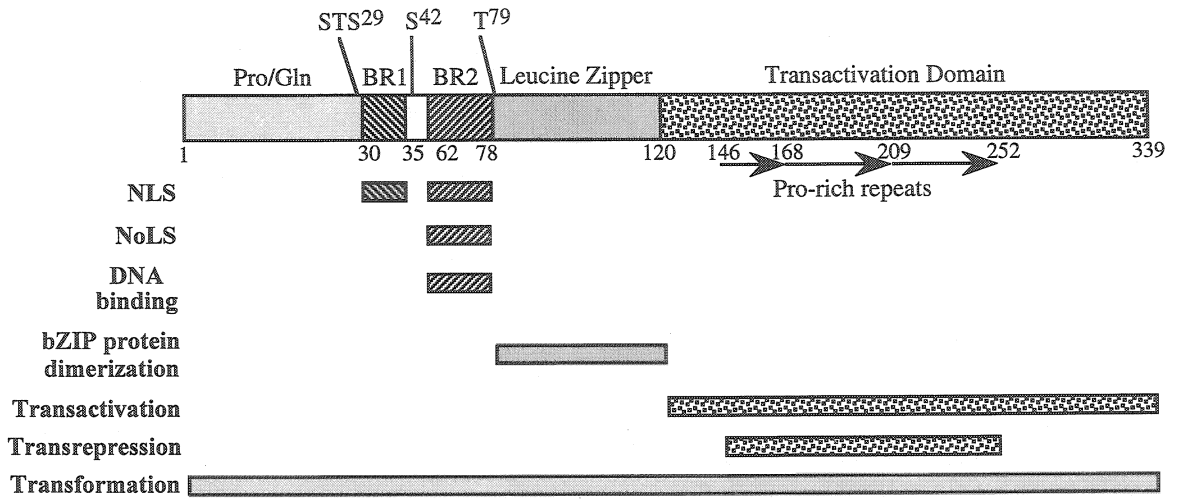

Fig. 1. Molecular structure, functional domains and major phosphorylation sites of MEQ oncoprotein.

replication, transcription/translation and immune responses. Indeed, the viral proteins often interact with the cellular network at more than one branch point to effectively, and timely, alter the host environ to suit their own purpose. As a consequence, the viral proteins can be viewed as a "highly genetically selected probe" to study the key cellular processes. One classical example is SV 40 large T antigen; investigation of cellular proteins interacting with SV 40 large T antigen have time and again led to the discoveries of key factors involved in cell cycle control, chromatin remodeling, transcription, tumorigenesis, etc. (reviewed in 1). In this review, we will focused on Marek's disease virus (MDV) oncoprotein, MEQ (2). As will be shown later, MEQ interacts with a large number of cellular proteins and affects several cellular processes.

Marek's disease virus (MDV), an avian alphaherpesvirus, is one of the most potent oncogenic herpesviruses. It has provided researchers with an excellent model to study the mechanisms involved in herpesviral oncogenesis and virus-host interactions. MDV infection elicits a rapid onset of malignant T-cell lymphomas in chicken within several weeks after infection (reviewed in 3–5). A recent report shows that MDV is able to transform chicken embryo fibroblasts (CEF) *in vitro* (6), although the evidence for sarcoma formation *in vivo* is still lacking. The short span of development and polyclonal nature of MDV-induced lymphomas suggest that one or more virally encoded oncogenes is directly involved in the transformation process. Several candidate genes encoded by the *Bam* HI D, H, I_2, L, and Q_2 fragments of the MDV genome have been implicated in oncogenesis (7–10). Among them, MEQ (*M*DV *E*co *Q*) is most consistently detected in all tumor samples and cell lines (2,10). MEQ encodes a 339-amino-acid protein with an N-terminal basic region-leucine zipper (bZIP) domain and a C-terminal transactivation domain (Fig. 1; 2). The bZIP domain, which consists of two stretches of basic residues (basic region 1 & 2) and a leucine zipper, shares significant homology with that of the Jun/Fos family of transcription factors and other families of bZIP proteins, such as CREB, Maf and SNF (Fig. 2). The highest homology is detected between MEQ and SNF, a protein which is comprised of primarily the bZIP domain (W. Wachsmann, personal communication). The transactivation domain is characterized by two and a half proline-rich repeats which contain several SH3 binding motifs. Although no cellular homologue has been defined for the transactivation domain, the high proline content of this domain is reminiscent of the transrepression domain of WT-1 (11). Thus, MEQ appears to be a fusion of a primordial bZIP protein (such as SNF) and a transactivating protein of unknown origin. MEQ is an exceptionally versatile protein, which apparently can localize to different

	Basic Region	Homology (%)
MEQ	EEEKQKLE RRRKRNRD AARRRRRK QTDYVDK	100.0
SNF	EDDDRKV- RRREKNRV AAQRSRKK QTQKADK	66.7
c-Jun	SQERIKAE RKRMRNRI AASKCRKR KLERISR	61.3
c-Fos	--EEEKRR IRRERNKM AAAKCRNR RRELTDT	48.3
CREB	--AARKRE VRLMKNRE AARECRRK KKEYVKC	55.2
v-Maf	--IRLKQK RRTLKNRG YAQSCRFK RVQQRHV	34.5

	Leucine Zipper	Homology (%)
MEQ	LHEACEE LQRANEH LRKEIRD LRTECTS LRVQLACH	100.0
SNF	LHEEYES LEQENIM LRREIGK LTEELKH LTEALKEH	44.4
c-Jun	LEEKVKT LKAQNSE LASTANM LREQVAQ LKQKVMNH	27.8
c-Fos	LQAETDQ LEEEKSA LQAEIAN LLKEKEK LEFILACH	36.1
CREB	LENRVAV LENQNKT LIEELKA LKDLCH- --------	33.3
v-Maf	LESEKNQ LLQQVEH LKQEISR LVRERDA YKEKYEKL	30.6

Fig. 2. Alignment of the protein sequence in the basic region and the leucine zipper between MEQ oncoprotein and other families of bZIP transcription factors.

cellular compartments and interact with a variety of cellular factors. The present review is divided into two sections each describing respectively the biochemical and biological properties of MEQ. Some of these novel properties are shared by other viral and cellular proteins. Whenever possible, comprehensive reference tables listing proteins with these properties are included to provide a proper context to understand the functions of MEQ.

1. Biochemical Properties of MEQ

Interaction with Cellular bZIP Proteins

A hallmark of bZIP proteins is their ability to become effective transactivators through dimerization with itself, or with other bZIP partners (12,13). Only dimer molecules can bind to their responsive elements and transactivate the target genes. By using a combination of *in vitro* co-immunopcecipitation, GST fusion protein precipitation, and electrophoresis mobility shift assays, MEQ has been found to dimerize with itself and to interact with a variety of bZIP proteins, including c-Jun, B-Jun, c-Fos, CREB, ATF-1, ATF-2, and SNF (14). In addition, MEQ can also form stable complexes with non-bZIP proteins, such as p53, pRb and MDV ICP4 (Brunovskis et al., unpublished results). Among them, the functional interaction with c-Jun has been studied in greater detail. MEQ-Jun heterodimer binds to an AP1-like sequence in the *meq* promoter region with a higher affinity than that of MEQ-MEQ or Jun-Jun homodimer (15). In addition, the double-staining immunofluorescence assays analyzed by an LSM confocal fluorescence microscope revealed that MEQ and activated (phosphorylated) c-Jun colocalize in the nucleoplasm (16). These data demonstrated that the interaction between MEQ and c-Jun occurs not only *in vitro*, but also possibly *in vivo*. The biological consequence of their interactions is discussed below.

Transcriptional Activation

The proline-rich region has been implicated as a domain involved in protein-protein interactions. It can be a simple short proline-rich sequence or a tandemly repeated sequence. These sequences have been identified in a variety of transcription machinery/regulators, including RNA polymerase II, WT1, CTF/NF1, and EBV EBNA 2 (17). In MEQ, there are two and one-half repeats of proline-rich sequences; more than 36% of amino acid residues within the repeat are prolines. To evaluate its transactivation potential, the entire C-terminal domain portion (amino acid 209–339) encompassing the proline-rich repeats of MEQ was fused to the yeast Gal4 (1–147) DNA-binding domain (15). Strong transactivation activity was observed. The last 33 amino acids at the C-terminus was found to be essential. At least one copy of the proline-rich repeats is also required to give full transactivation activity. Interestingly, the proline-rich repeat in its isolated form (i.e. in the absence of other MEQ c-terminal sequence) exhibit strong transrepressing activity, in a manner similar to the proline-rich sequence of WT-1 tumor suppressor protein. The fact that MEQ contains subdomains with both transactivating and transrepressing functions suggest that MEQ may have both properties, depending on the phosphorylation state, the interacting partners, or other factors that modulate the conformation of MEQ. Wild type MEQ, in the presence of c-Jun, is a potent transactivator on TRE (TPA Response Element)/CRE (Cyclic AMP Response Element)-driven promoter (15). The promoter of *meq* gene itself contains a TRE/CRE hybrid sequence and can be transactivated by MEQ/Jun heterodimer. These data provide the first biochemical evidence that MEQ is a transcription factor and c-Jun is one of MEQ's interacting partners. Using a TRE/CRE-driven reporter gene as an assay in conjunction with the various C-terminal deletion mutants, the transactivation potential of the C-terminal domain in the context of the wild type MEQ was recently examined (15). The data is consistent with that

derived from the Gal-MEQ fusion proteins in that the C-terminal domain contains transactivation function and at least one of the proline-repeats is required. Data in support of the transactivation function of the C-terminal domain also come from the studies of the MEQ splicing variants (18 and Li et al., unpublished results). One variant, MEQ-sp, which skips the entire C-terminal domain does not transactivate and in fact serves as a dominant-negative molecule for the wild type, presumably by forming inactive dimer. A second variant in which the last 9 amino acids at the C-terminus are replaced by other viral sequences is also defective in transactivation. The above data suggests that MEQ is a modular protein, with the N-terminal half involved in DNA binding, dimerization and nuclear transport, and the C-terminal half in transactivation. The question that needs to be answered in the future is the nature of transcription factors or co-activators the C-terminal domain interacts with.

Target DNA Response Elements

In an effort to identify viral and cellular targets for MEQ, PCR-based approach, CASTing (cyclic amplification of selected sequences; 19) was used. MEQ-Jun heterodimers were found to optimally bind to TRE and CRE consensus sequences. This result was consistent with that of reporter assays using TRE/CRE-driven promoter, described above. On the other hand, MEQ-MEQ homodimers were shown to bind to two distinct response elements, namely MERE (MEQ Response Element) I and MERE II (20). MERE I [GAGTGATGAC(G)TCATC] is similar to TRE/CRE motif, except with extension at both 5′ and 3′ ends. These extended sequences are found to be critical for tight binding of MEQ/MEQ homodimer to MERE I. Methylation interference analysis, using *in vitro* translated MEQ, confirms that the flanking residues are protected by MEQ/MEQ dimer. These sequences however are not well protected by Jun/Jun which recognizes the central TRE/CRE core motif. These data indicate that the bZIP domain of MEQ/MEQ are conformationaly different from Jun/Jun, with the former having a more extended conformation than the latter. Interestingly, Maf, another bZIP oncoprotein of aivan retrovirus, also recognizes a motif similar to MERE I (21). Whether MEQ functionally interacts with Maf remains to be elucidated, but it is conceivable that MEQ may utilize the Maf pathway in cellular transformation. The second putative MEQ binding site, MERE II (RACACACAY), bears a completely different consensus motif not shared by other bZIP proteins. Not all CACA motif binds MEQ-MEQ dimer and those which bind, seem to have a higher curvature (20). CACA motifs are known to promote DNA curvature and function in a number of special biological processes (22,23). A potential target bearing the CACA motif is found near the replication origin of MDV and shown to bind MEQ-MEQ dimer (Brunovskis et al., unpublished results). This replication origin serves a dual role as a divergent promoter of viral genes pp38 and pp14. It would be of interest to determine whether the binding of MEQ homodimer in this region is involved in the regulation of MDV replication and transcription. The above results showed that MEQ-MEQ dimer assumes a conformation distinct from Jun-Jun, Jun-Fos or Jun-MEQ heterodimer, and is expected to activate a set of genes different from other Jun/Fos family proteins. On the other hand, the presence of other Jun/Fos family proteins would favor heterodimer formation of MEQ, which recognize a more convenetional TRE/CRE site and thus significantly affect the type of genes regulated.

RNA-Binding Activity

We previously noted that MEQ contains an RNA-recognition motif (RNP-1) at the C-terminus, in addition to an arginine-rich region (arginine-fork) which is highly homologous to the RNA binding domains identified in many viral proteins including human T-cell leukemia virus-I (HTLV-I) Rex, human immunodeficiency virus-I (HIV-I) Rev and Tat, herpes simplex virus U_s11, hepatitis Delta antigen etc. (Table 1; 24–30). Many of these viral proteins, e.g. Rev, Rex and U_s11 are involved in the regulation of viral RNA processing or transport. Preliminary results indicate that MEQ indeed associates with total cellular RNA (Liu et al., unpublished results), although whether there is any binding specificity remains to be determined. Likewise, the biological significance of the RNA-binding properties of MEQ remains unknown. Whatever the function of the RNA-binding potential of MEQ is, its association with rRNA may account for MEQ's ability to transport into the nucleolus (see below), a site for ribosomal RNA biogenesis. MEQ's ability to bind RNA is unique among all Jun/Fos family bZIP proteins. This may

Table 1. Alignment of the arginine-fork RNA binding motif identified in viral proteins

Viral protein	RNA binding motif	Reference
HTLV-I Rex	MPKT**RRRPRRS**QR**KR**PPTP	24
HIV-I Rev	T**RQARRNRRRR**WR**ER**Q**R**	25
HIV-I Tat	ALGISYG**RKKRRQRRR**P	26
Hepatitis Delta	EK**RQDHRRR**KA	27
antigen	EDEK**RERR**IAG	
HBV Capsid protein	**RRRDRGR**	28
BMV Gag	KMT**RAQRRAAARRNR**WTA**R**	29
CCMV Gag	KLT**RAQRRAAAR**KNK**R**NT**R**	29
λ N	MDAQT**RRRERR**AEKQAQW	30
φ 21N	GTAKS**RYKAR**AELIAE**R**	30
P 22N	GNAKT**RRHERRR**KLAIE**R**	30
MDV MEQ	**RRRKRNRDAARRRR**KQT (?)	

contribute to the overall different immunostaining pattern of MEQ and JUN (16), even though some of these proteins are colocalized in the nucleoplasm.

Phosphorylation

In response to cellular signals, transcriptional factors are often regulated by phosphorylation at the levels of DNA-binding, transactivation and nuclear import/export (reviewed in 31 & 32). A multitude of serine/threonine kinases participates in this type of regulation. For instance, Jun has been shown to be phosphorylated by a sleuth of serine/threonine kinases, e.g., PKC (33), JNK/SAPK (34), MAPK (35), CKII (36), CDK1 (33), DNA-PK (37), GSK3 (38), and c-Raf-1 (39), at different serine/threonine residues, resulting in different biochemical properties of c-Jun.

Likewise, MEQ is also a phophoprotein *in vivo* and serves as an excellent substrate for PKA, PKC, MAPK, CDK, CKII by *in vitro* kinase assays. The primary phosphorylation sites have been mapped to STS29 (PKC), S42 (CDK), and T79 (PKA), which lie adjacent to the basic regions (see Fig. 1). The EMSA (Electrophoretic Mobility Shift Assay) results showed that phosphorylation of MEQ oncoprotein by PKC enhanced its DNA binding activity. By contrast, phosphorylation by CDK drastically reduced its DNA binding activity. In addition, the cell cycle-dependent cytoplasmic translocation of MEQ oncoprotein seems to be regulated by CDK2 phosphorylation during S phase. The biological relevance of CDK phosphorylation of MEQ is discussed in detail below.

2. Biological Properties

Subcellular Localization

The physiological and pathological functions of viral and cellular proteins are often reflected by their subcellular localizations, particularly in the specific subnuclear organelles. As exemplified by the herpesvirus ICP4 gene product (40–42), viral proteins tend to be more "mobile" than cellular proteins, perhaps allowing them to interact with different cellular factors at different organelles during different phases of viral replication. MEQ has been shown to express in the nucleus, but interestingly, with a predominant fraction in the nucleoli and coiled bodies (43). This makes MEQ the first bZIP protein to be identified in the nucleoli. MEQ contains two stretches of basic residues, designated as basic region 1 (BR1) and basic region 2 (BR2). Using a series of deletion mutants, we have mapped the primary nuclear localization signal (NLS), and the sole nucleolar localization signal (NoLS), to the BR2 region (43). When fused to cytoplasmic protein v-Raf,

BR2 is able to translocate v-Raf into nucleoplasm and nucleolus, indicating BR2 is not only a necessary but also a sufficient nuclear and nucleolar localization signal. The BR2 region can be further divided into two long arginine/lysine stretches, BR2N and BR2C, which are separated by the five amino acids Asn-Arg-Asp-Ala-Ala (NRDAA). Both sequences are required for nucleolar localization, whereas either subdomain alone is sufficient for nuclear localization, indicating that the requirement for nucleolar translocation is more stringent than that for nuclear translocation. Consistent with this observation, BR1 is able to signal only nuclear, but not nucleolar, localization.

Given MEQ's role as a transcription factor, its nucleolar and coiled body localization is somewhat perplexing. However, this is by no means unique; a number of transcription factors such as YY1 (44), HOX B7, C6, and D4 (45), the tumor suppressor pRb (46), and the TATA-binding protein (TBP) (47,48) are also found in the nucleolus, whereas WT-1 is found in the coiled bodies (49). An extended list of nuclear proteins that have multiple localization in subnuclear organelles is shown in Table 2. Several viral proteins such as HTLV-1 Rex (50), HIV Rev (51–54) and HIV Tat (55,56) which bind RNA are also in the list. In the herpesvirus kingdom alone, at least three, HSV-1 U_S11 (57), infected cell protein 27 (ICP27) (58,59) and EBNA5 (60,61) are known to localize to the nucleolus. Given the large number of proteins that exhibit these properties, it is perhaps to be expected that their functions in special subnuclear organelles are quite diverse. For instance, TBP and pRb, well known for their functions in transcriptional regulation

Table 2. Multiple subnuclear compartmentalization of cellular and viral transcription factors

Protein	Nucleoplasm	Nucleolus	Coiled Body	Spliceosome	PML	References
Cellular						
YY1	+	+				44
HOXB7, C6, D4	+	+				45
pRb	+	+				46
TBP	+	+				47, 48
WT1	+		+	+		49
PCNA	+	+				64
HSP70	+	+				66
LYAR	+	+				67
IFI 16	+	+				68
DSSRP	+	+				
Viral						
MEQ	+	+	+			43
Rex	+	+				50
Rev	+	+		+		51-54
Tat	+	+				55, 56
Us11	+	+				57
ICP27	+	+		+		58, 59
EBNA-LP	+	+			+	60, 61
IVa2	+	+				69
E1A	+				+	70
E4-ORF3	+				+	70
ICP0	+				+	71
Tax	+			+		72

by RNA polymerase II, regulate rRNA transcription in nucleolus. Similarly, the nucleolar localization of Rev (62) and Rex (50), is required for their functions in posttranscriptional regulation of viral mRNA, so is herpes simplex viurs U_s11 (63). The nucleolar and coiled-body localization of MEQ may thus foretell a function beyond its role as a transactivaator for Pol II transcripts.

Transforming Potential

As mentioned in the introduction, *Meq* is the most consistently detected MDV gene in all tumor samples and cell lines. Xie et al. (73) used an antisense strategy to show that *meq* is required for the maintenance of the transformed state of an MDV tumor cell line, MSB1. As there is no efficient chicken *in vitro* T-cell transformation system available, fibroblast cell lines were used to explore MEQ's mitogenic properties.

Over-expression of MEQ by means of retroviral infection results in transformation of a rodent fibroblast cell line, Rat-2. The criteria for transformation include morphological transfiguration, anchorage-independent growth and serum-independent growth (74). When expressed at a lower level (as was the case in cells transfected with MEQ DNA), MEQ-mediated transformation requires a complementing oncogene such as v-*ras*. In this assay, c-Jun behaves similarly to MEQ, in that it also requires v-Ras for full transformation phenotypes of Rat-2 (75). Recent studies by Castenollazi (76) indicated that Jun-Fos partnership is responsible for serum-independent growth and Jun-ATF2 partnership for anchorage-independent growth of Jun-transformed cells. v-Ras is known to activate MAPK/Elk pathway which lead to Fos activation. Overexpression of Jun would facilitate the pairing not only with Fos, but also with ATF2, a transcription factor activated by the SAPK and p38/mHOG, which may account for the complementarity of Jun and Ras. Given the ability of MEQ to dimerize with Fos and ATF2, it is possible that MEQ transforms cells by taking the same path as Jun. Using the v-ras-complementation assay, the transformation domains of MEQ were delineated. We found the minimal transformation could be achieved by the MEQ bZIP domain with BR2 region being absolutely essential, as it is the major nuclear localization signal and the DNA binding domain. For full transforming activity, the C-terminal transactivation domain was also required, suggesting that transactivation function plays an important part in MEQ-mediated transformation. To study whether the transformation potential of MEQ may mediate through the C-Jun pathway, the bZIP domain of Jun was used to replace that of MEQ to generate a chimeric construct, Jun (bZIP)-MEQ (TA). This construct is able to complement v-ras in the transformation assay. Likewise, the c-Jun transactivation domain can also functionally substitute for that of MEQ (75). Taken together, these data suggest that at least in this transformation assay, the bZIP and transactivation domain of c-Jun can functionally replace those of MEQ.

Antagonism of Apotosis

To maximize the yield of progeny virus, or to establish latency, viruses have evolved a variety of ways to block apoptosis of virus-infected cells (see Table 3). Some viruses encode Bcl-2 homologues to mimic the anti-apoptotic function of cellular Bcl-2, such as EBV BHRF-1 (77). Others encode viral proteins, such as adenovirus E1B-55K (78), which can inhibit apoptosis through sequestration of the p53 protein known to trigger apoptosis. There are also viruses which encode Fas/TNF-R antagonists to interfere with the signaling pathways involved in apoptosis, such as baculovirus LAP (79) and viruses which encode protease inhibitor to directly abolish the function of caspases, such as cow poxvirus *crm*A (80). Finally, some viruses encode transcription factors to transregulate genes involved in apoptosis and/or cell survival, such as HSV-IICP4 (81).

MEQ is a transcription factor and directly interacts with p53. It protects Rat-2 cells from cell death induced by multiple modes including TNF-α, C2-Ceramide, UV irradiation, and serum deprivation (74). Its anti-apoptotic function requires new protein synthesis, as treatment with a protein synthesis inhibitor, cycloheximide, partially reversed MEQ's anti-apoptotic effect. Coincidentally, transcriptional induction of *bcl*-2 and suppression of *bax* are also observed in MEQ-transformed Rat-2 cells. It is not clear whether this modulation is through direct binding of MEQ to the promoter of these genes, or through other factors activated by MEQ. We note that MEQ binding sites (MERE-I and -II) are found in the promoter of the human *bcl*-2 gene. Alternatively, the up-regulation of Bcl-2 and down-regulation of Bax observed in MEQ-transformed Rat-2 cells might be

Table 3. Mechanisms of viral proteins involved in antagnosis of apoptosis

Mechanism	Virus	Protein	Reference
Bcl-2 homolog	EBV	BHRF-1	77
	Adenovirus	E1B-19K	82, 83
	Herpesvirus saimiri	ORF16	84
	HHV-8	KSbcl-2	85
	ASFV	LMW5-HL	86, 87
p53 sequestration	Adenovirus	E1B-55K	78
		E4orf6	88
	HPV	E6	89, 90
	SV-40	Large T antigen	91, 92
	Hepatitis B virus	pX	93
	EBV	EBNA-LP (?)	94
	EBV	ZEBRA (?)	95
	MDV	MEQ (?)	
Fas/TNF-R antagonist	Baculovirus	IAP	79
	Adenovirus	E3	96
	EHV-2	E8	97, 98
	Myxovirus	T2, M11L	99
	ASFV	A224L	100
	Gamma Herpesvirus	vFLIPs	101
Protease inhibitor	Cow poxvirus	*crm*A	80
	Baculovirus	p35	102
	Vaccinia virus	SPI-2	103
Transregulation of genes involved in apoptosis and/or cell survival	MDV	MEQ	74
	HSV-I	ICP4	81
	EBV	LMP1	104
	CMV	IE1, IE2	105

mediated by sequestering p53 through interactions. It is also possible that other Bcl-2- and Bax-like molecules are regulated by MEQ as well. Taken together, our results suggest that MEQ antagonizes apoptosis through regulation of its downstream target genes involved in apoptotic and/or anti-apoptotic pathways.

Deregulation of Cell Cycle Progression

Cyclin-dependent kinase 2 (CDK2) is usually localized through out nucleoplasm, where they act to inactivate pRb protein (106). In MEQ-transformed cells, there is a cell cycle-dependent colocalization of MEQ protein and CDK2 in coiled bodies and the

Table 4. Mechanisms of deregulation of host cell cycle control engaged by viral proteins

Mechanism	Virus	Protein	Reference
Transregulation of cell cycle regulatory genes	Adenovirus 2	?	110
	SV-40	?	111
	HPV-16	E7	121
Cyclin homologue	HSV-I	v-cyclin	112
	HHV-8	v-cyclin	113
Rb sequestration	SV-40	Large T antigen	114
	Adenovirus	E1A	122
	HPV-16	E7	123
	EBV	EBNA-3C	124
	CMV	IE1, IE2	125, 126
	MDV	MEQ	
p53 sequestration	SV-40	Large T antigen	115
	Adenovirus	E1B	127
	HPV-16	E6	128
	CMV	IE2, *mtr*II	129, 130
	MDV	MEQ	
Interaction with CDK-cyclin			
CDK2	MDV	MEQ	107
CDK2-cyclin A	SV-40	Large T antigen	116
Cyclin D3	HSV-I	ICP0	131
	HTLV-I	Tax	132
CDK7	HIV-I	Tat	133, 134
CDK9-cyclin T	HIV-I	Tat	135
CDK2-cyclin A, E	HPV-16	E7	136
CDK8	HTLV-I	Tax	137
CDK8	Adenovirus	E1A, VP16	138
Inactivation of CDK inhibitors			
P16INL4A	HTLV-I	Tax	117, 118
P21WAF1	HTLV-I	Tax	119
	HPV-16	E7	139
P27KIP1	HPV-16	E7	140
	Adenovirus	E1A	141
Translocation of CDK-cyclin	MDV	MEQ	107
	CMV	?	120

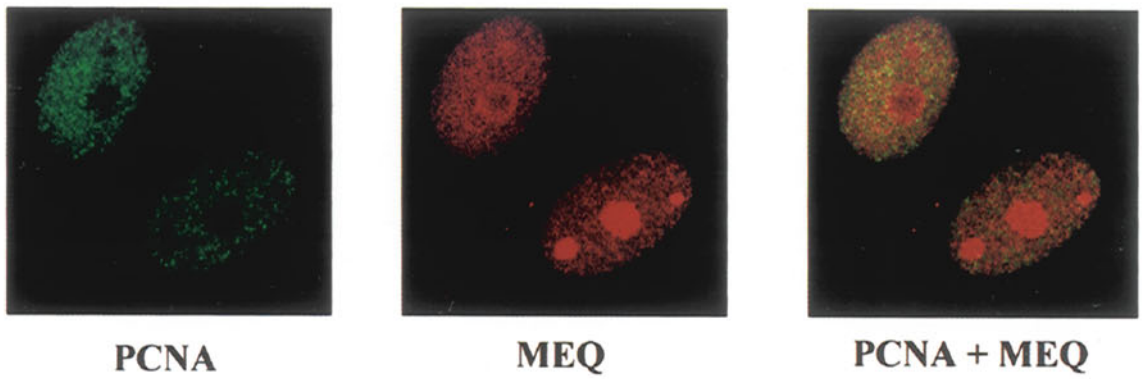

Fig. 3. Colocalization of MEQ oncoprotein with PCNA in the nucleoplasm. MEQ-transformed Rat-2 cells were double-stained with anti-PCNA MAb (1: 100 dilution) and anti-MEQ polyclonal antibodies (1: 400 dilution) followed by FITC- and Texas red-conjugated secondary antibodies. The fixed cells were analyzed with a LSM confocal microscope.

nucleolar periphery during early S phase (107). To our knowledge, this is the first report that CDK2 is localized to the coiled bodies. What is the significance of this colocalization? This allows CDK2 to phosphorylate MEQ, which may account for MEQ's translocation into the cytoplasm in S phase. MEQ is found in both the nucleus and the cytoplasm during S phase, and MEQ can be phosphorylated *in vitro* by purified CDKs at reseidue 42 serine. An indirect immunofluorescence study of the MEQ mutant, S42D, in which serine 42 was mutated to a charged residue to simulate phosphorylation, reveals more prominent cytoplasmic localization. Furthermore, phosphorylation of MEQ by CDKs drastically reduces the DNA-binding activity of MEQ, which may in part account for the lack of retention of MEQ oncoprotein in the nucleus. Conversely, MEQ may play a role in CDK2's translocation into the coiled bodies and the nucleolar periphery, since the localization of CDK2 in these regions is observed only in MEQ-transformed Rat-2 cells. What then is the consequence of CDK2's translocation into the coiled bodies? One potential consequence is the increased accessibility of CDK2 to CDK7-cyclin H, which in turn may phosphorylate pRb, resulting in cell-cycle deregulation.

If MEQ indeed is responsible for CDK2 translocation, with consequential cell cycle deregulation, it joins a growing list of DNA tumor virus oncoproteins which utilize deregulation of cell cycle as a strategy to transform host cells. In uninfected cells, the cell cycle is controlled by the activity of CDK-cyclin complexes. To deregulate cell cycle, components linking to CDK-cyclin complexes are usually targets for the viruses. The strategies are summarized in Table 4, and briefly reviewed here (108,109). First, adeno-associated virus (Ad) type 2 (110) and simian virus (SV)-40 (111) encode transcription factors that modulate the expression of cell cycle regulatory genes. Second, some viruses encode cyclin homologues on their own, such as the v-cyclin of HSV1 (112) and HHV8 (113), to subvert the cell cycle regulation. Third, some viral proteins, such as SV-40 large T antigen, sequester tumor suppressor proteins such as pRb (114) or p53 (115). As mentioned above, MEQ is capable of binding p53. But in addition, it contains a LXCXE motif and interacts specifically with pRb (Brunovskis et al., unpublished results). Fourth, some viral proteins interact with and stabilize CDK/cyclin complexes, as SV40 large T antigen does with CDK2/cyclin A (116). Fifth, some viral proteins inactivate CDK inhibitors by the formation of an inactive complex, as p16INK4A (117,118) and p21WAF1 (119) are inactivated by HTLV-1 Tax. And finally, CMV, in a manner similar to MDV, induces the nuclear translocation of CDK2 in serum-starved and contact-inhibited cells (120), although translocation into coiled bodies is not reported. It thus seems deregulation of host cell-cycle progression is a common and crucial step during the transformation processes by DNA tumor viruses. MEQ's ability to interact with CDK2 in coiled bodies adds yet another clever strategy.

DNA Replication

The role of MEQ involved in the DNA replication is presently unknown. However, two pieces of evidence may implicate MEQ in regulation of MDV viral and/or host DNA replication. First, there is a MERE II (RACACACAY) motif located adjacent to the replication origin of MDV 1 and the binding site for UL9, a viral DNA binding protein involved in MDV replication. It has been shown that MEQ-MEQ homodimers binds to this region with a great affinity. Thus MEQ might interfere with MDV viral DNA replication by preventing the binding of the DNA replication machinery to its replication origin. This concept is consistent with the observation that MEQ is constitutively expressed in MDV tumor cell lines where little viral replication takes place. Secondly, double-staining immunofluorescence assays showed that MEQ colocalizes with PCNA in the potential DNA replication folks (Fig. 3). This finding suggests that MEQ may be directly involved in the DNA replication process.

Epilogue

In summary, we describe here a viral oncoprotein which is structurally similar to the bZIP transcription factors, yet, function-wise, it is much more versatile and interacts with a sleuth of cellular factors (Fig. 4). By establishing itself as an analogue of cellular genes, such as c-Jun, MEQ is able to readily engage itself

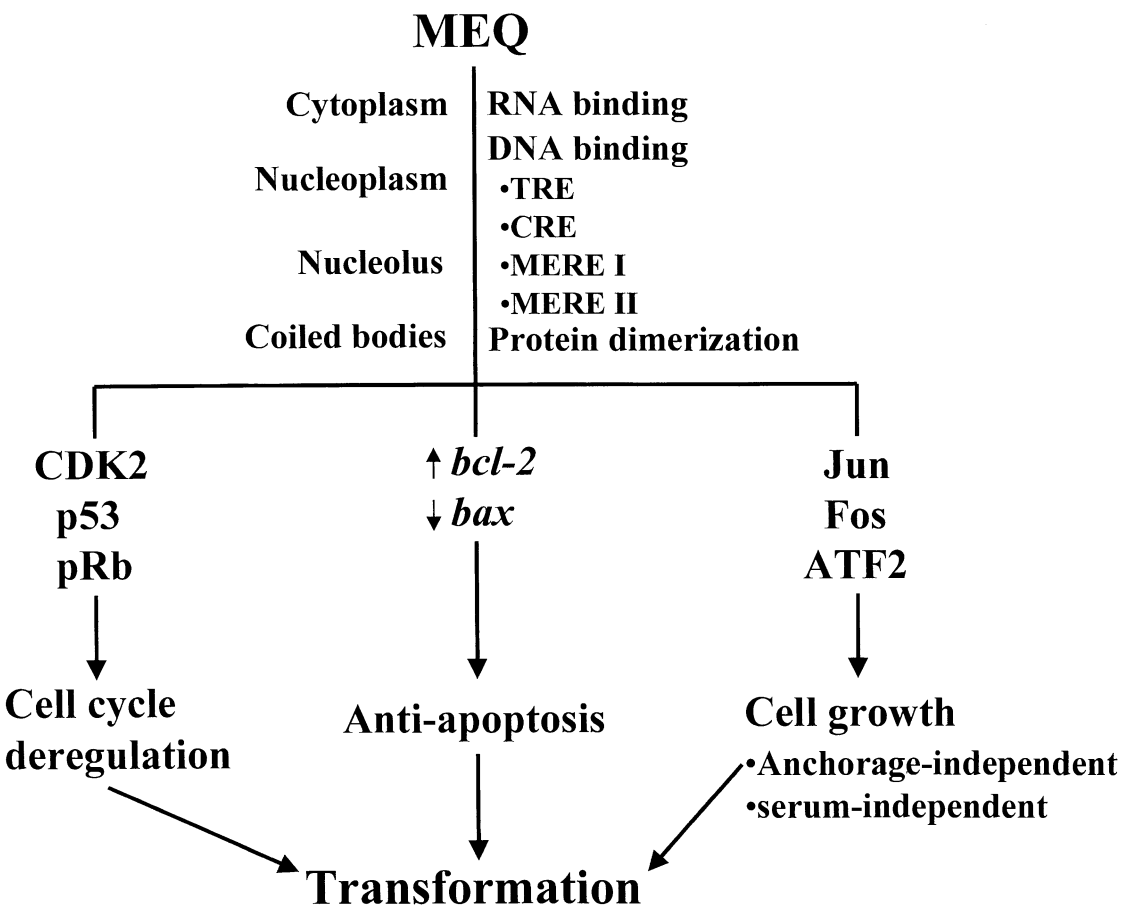

Fig. 4. Summary of the versatile functions exhibited by MEQ oncoprotein through different subcellular localization and interaction with various cellular factors.

with the signal pathways leading to growth and transformation, presumably via transcriptional (de)regulation of host genes. However, MEQ also exhibits novel biological functions that go beyond c-Jun, including binding to RNA, binding a CACAC motif, binding p53 and pRb, interaction with CDK2, and inhibition of apoptosis. Perhaps necessitated by its interactions with a variety of cellular factors, MEQ has multiple subcellular localizations including cytoplasm (in S phase), nucleoplasm, nucleolus and coiled body. Why does MEQ have to perform so many different functions? Is this an overkill? Perhaps, and perhaps not. It is true that some of the functions were observed in specific cell types *in vitro* and have yet to be confirmed *in vivo*. On the other hand, MEQ is likely to play a dual role as a replication and a transforming protein for MDV. Some of the observed functions of MEQ may actually be required for replication and survival of MDV *in vivo*. The recent successful isolation of a MEQ-negative mutant of MDV by targeted deletion (142) will surely shed significant light to this question. The MEQ-negative mutant appears to be completely non-oncogenic; it replicates well *in vitro* but only transiently *in vivo*. There are numerous examples of virus proteins that perform both replication and transforming functions. As illustrated in the Tables presented in this review, antiapoptosis and cell-cycle deregulation appear to be common traits for many virus proteins, through which we have come to learn a great deal about these processes. MEQ may provide yet another paradigm to understand them.

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Herpesvirus Homologues of Cellular Genes

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Abstract. For millions of years viruses have adapted strategies to interfere with the immune defense of the host, which in turn has to deal with this challenge. In general the antiviral defense remains one step behind the pathogen. To achieve this strategic advantage large DNA-containing Viruses encode cellular homologues that mimic or counteract key molecules of the host immune system. Understanding how these cellular homologues enable the viruses to evade the antiviral defense and persist in the host for the lifetime will ultimatively lead also to a better understanding of the principle functions of the immune system. In this review we focused on cellular homologues encoded by human herpesviruses and discuss the functional consequences of their expression.

Key words: herpesviruses, cellular homologues, immune evasion, viral evolution

Introduction

The family *Herpesviridae* are enveloped dsDNA viruses that are subdivided into three subfamilies, *alpha, beta* and *gammaherpesvirinae*, on the basis of conserved biological features. Eight of almost a hundred known herpesviruses can infect humans, including representatives from each of the three subfamilies. Herpesviruses have one of the largest viral genomes, with approximately 200 kb of DNA encoding over a hundred genes. Within this wealth of genetic material are many genes that the virus has pilfered from its host. There is plentiful evidence that the use of homologues of host proteins is important in the evolution and life cycles of viruses, and in few other viral groups are there so many homologues found as in the *Herpesviridae*. In this review we attempt to summarize all herpesviral proteins that bear a known homology to cellular proteins involved in immunity. We have excluded the plentiful viral homologues that are not directly concerned with immunity, although as discussed later they may be responsible for inappropriate immune responses. In assessing homology we have attempted to relate structural homology to conservation of function, and the significance of changes therein which promote viral immune evasion. We have also chosen to focus on the eight human herpesviruses, and have included herpesviruses from other species only in so far as they illustrate specific examples of viral piracy from Homo Sapiens. Therefore all comparisons given are drawn from human host proteins and homologues given for animal herpesviral proteins relate to the closest related human herpesvirus protein. In evaluating the degree of identity between proteins we utilized the BLAST suite of programs (1) to generate a coherent overview of homology and the latest released sequence data. The Clustalx program (2) was employed to perform multiple alignments and the SMART suite of programs (3) was used to identify transmembrane domains by the method of Lupas et al. (4).

Viral homologues of cellular proteins that are involved in immunity can be categorized into the following groupings: chemokines, cytokines, apoptosis-related genes, the complement system, Fc-receptors and immunoglobulin superfamily proteins. It appears that in relation to immunity whether a particular virus encodes a homologue of a cellular protein is a question of ancestry and chance. In other words related herpesviruses often contain similar homologues of cellular proteins, indicating the conservation of a successful immune evasion strategy

acquired by chance. To evade the various effector mechanisms of the immune system herpesviruses often employ multiple homologue-based strategies. These shape the viral life-cycle and pathogenicity within its host.

Chemokines and Chemokine Receptor Homologues

Chemokines are small soluble molecules that induce chemotaxis of certain leucocyte populations. They are divisible into four groupings by their possession of conserved cystein motifs, the motifs being CXC, CC, C or CX_3C (where X represents any amino acid). The CXC and CC groups are referred to as α and β chemokines respectively, and these two groups represent over 95% of all known chemokines. Chemokine receptors are typically G-coupled receptors (GCR) with a characteristic structure of seven membrane spanning domains. The receptors frequently can bind several different ligands, although these chemokines are usually from the same group. Receptors therefore are categorized by the group of chemokines they bind, for example CCR1 refers to β chemokine receptor 1. Functionally they may either be activated by binding to ligand or may be constitutively active. Herpesviruses often possess several chemokine receptors, in particular the *betaherpesvirinae*, human cytomegalovirus (HCMV) encoding four open reading frames that bear homology to GCR: UL33, US27, US28 (5) and UL78. All herpesviral chemokine or chemokine receptor homologues thus far found show strongest homology within either the α or β groups. We have summarized the degree of identity they show with their cellular counterparts in Table 1.

The GCR encoded in HCMV by UL33 is representative of a group of similar GCR homologues conserved between human and animal *betaherpesvirinae*. This group consists of human herpesvirus 6 (HHV6) U12, human herpesvirus 7 (HHV7) U12 (6), HCMV UL33, murine cytomegalovirus (MCMV) M33 and rat cytomegalovirus (RCMV) R33. The UL33 group show strongest homology to β chemokine receptors (CCR), with strong conservation of both individual amino acids and secondary structure (transmembrane domains) distributed across the entire protein (Fig. 1). The individual viral homologues within the UL33 group show greater identity with each other than to the closest known cellular homologue (Table 2) and this conservation can be used to draw a plausible dendrogram of the UL33 group (Fig. 2). The putative phylogeny demonstrated by Fig. 2 corresponds well to the level of general homology between the various members of the *betaherpesvirinae*. Little is known of their function or ligand specificity although it would appear obvious from their conservation that they are important. Recent research demonstrating that the murine (7) and the rat (8) homologues are essential for efficient infection and replication in salivary glands, in conjunction with the discovery that UL33 can be found on the virion itself is suggestive (9) in the light of chemokine receptors as gateways for viral entry. HCMV was originally described as a salivary gland virus (10), and the identification of a gene essential to this function conserved across the *betaherpesvirinae* may indicate the principal mode of transmission for betaherpesviruses. A second group of GCR homologues is found exclusively in HCMV. US28 and US27 strongly resemble each other and are most homologous to β chemokine receptors (Table 1), however unlike the UL33 group both are most

Table 1. Chemokine receptor homologs

Group	Viral Homolog	Familial Resemblance	Closest Cellular Protein	BLAST Identity	Accession Number
UL33 homologs	HCMV UL33	CC Receptor	CCR1	24%	P16849
	HHV6 U12	CC Receptor	CCR5	21%	X834183
	HHV7 U12	CC Receptor	CCR7	20%	P52381
US28 homologs	HCMV US28	CC Receptor	GPR13	35%	P09704
	HCMV US27	CC Receptor	GPR13	27%	P09703
Opioid Receptor	HCMV UL78	Opioid Reptor	SS3R	26%	P16751
homologs	HHV6 U51	Opioid Reptor	KOR-1	21%	P52382
	HHV7 U51	Opioid Reptor			U43400
CXC Receptor homologs	HHV8 orf 74	CXC Receptor	CXCR2	24%	Q98146

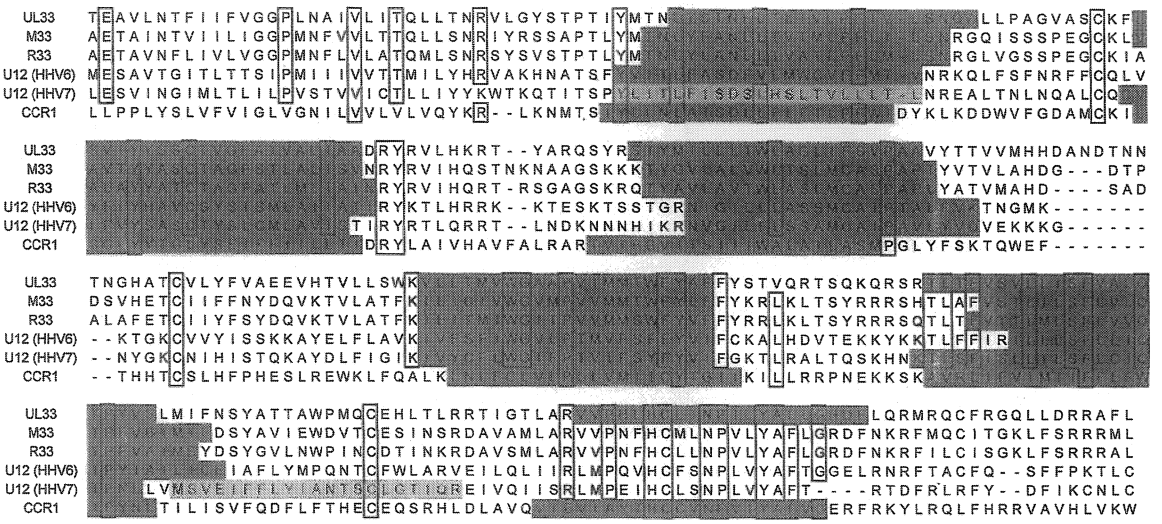

Fig. 1. Conservation between herpesviral GCR-homologues of the HCMV UL33 group and cellular CCR1. Boxing indicates identity between four or more homologues as determined by the Clustalx program. Dark shading denotes transmembrane domains, lighter shading indicates putative transmembrane domains as predicted by the SMART suite of programs.

homologous to the N-terminal residues which are known to be essential for ligand binding (11). US28, which has stronger homology to cellular proteins than US27, has been shown to be a functional receptor and can both bind β chemokines (12) and transduce signal into the cell (13). US28 expressed by HCMV-infected fibroblasts has also been shown to sequester chemokine into infected cells *in vitro* (14), thereby reducing chemokine concentration in surrounding medium. US28 can bind many β chemokines, including macrophage inhibitory protein (MIP)-1α, MIP-1β, RANTES (Regulated on Activation, Normal T cells Expressed and Secreted), monocyte chemotactic protein (MCP)-1 (12,13) and MCP-3 (14). Surprisingly it can also bind chemokines outside the β chemokine group. Fractalkine, the only known member of the membrane-bound CX_3C family of chemokines, has been shown to bind with high affinity to US28 (15).

Table 2. UL33 Chemokine receptor family

Homology to UL33	BLAST Identity	Accession Number
MCMV M33	47%	L41868
RCMV R33	47%	U91788
HHV6 U12	25%	X834183
HHV7 U12	24%	P52381
CCR1	24%	P32246

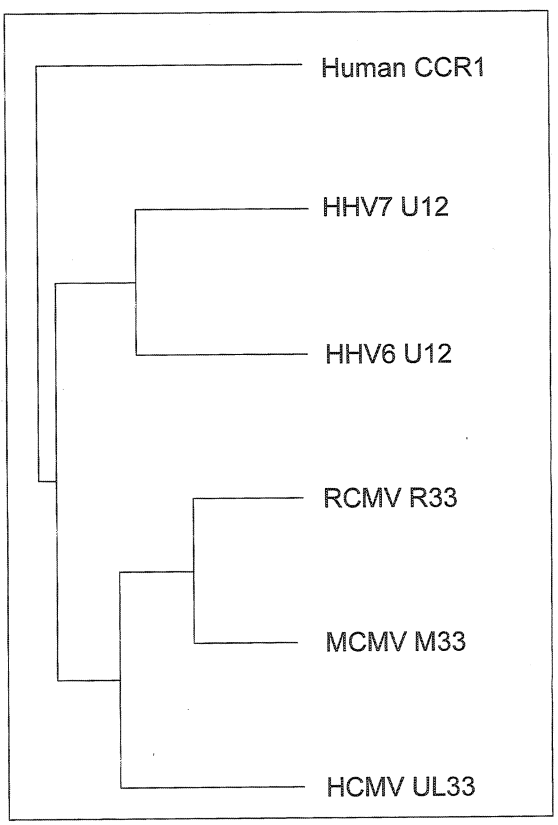

Fig. 2. Dendrogram showing relatedness of the UL33 group of chemokine receptors as calculated by the Clustalx program.

A third group of GCR homologues is represented on HCMV by UL78. This group is positionally conserved between animal and human betaherpesviruses but the individual members share less sequence homology with each other or with cellular proteins, and perhaps for this reason are less well characterized. Although showing poor homology with each other the members of this group consistently share homology with opioid receptors, and therefore we refer to them as the opioid group of GCR homologues (Table 1). The group consists of HHV6 U51, HHV7 U51, HCMV UL78, MCMV M78 (16) and RCMV R78 (accession number AF077758). HHV7 U51 has very weak homology to cellular opioid receptors, and has thus no BLAST score in Table 1, although it does show 37% homology to HHV6 U51. Functionally these homologues remain uncharacterized to date. One human gammaherpesvirus, HHV8, encodes a GCR on orf74 that is homologous with α chemokine receptors (CXCR) (17). It can bind and be activated by IL8 and growth-related protein (GRP)-α, although it also has constitutive activity independent of the presence of ligand (18,19). Several members of the animal *gammaherpesvirinae* encode α chemokine receptor homologues, including murine gammaherpesvirus 68 (20) and herpesvirus salmiri which encodes a functional GCR named ECRF3 that can bind IL-8 and other CXC chemokines and induce calcium flux (21,22).

In comparison to the number of chemokine receptor homologues there are fewer examples of chemokines encoded by herpesviruses (Table 3). HHV8 encodes three β chemokines, on orf K4 (vMIP-II), orf K6 (vMIP-I), and on orf BCK. As their name suggests, vMIP-I and vMIP-II are most homologous to MIP-1α (23,24), vMIP-I binds to similar receptors as endogenous MIP-1α, but vMIP-II is more promiscuous, in addition binding to CCR2 and CXCR4 (25). vMIP-II is able to bind to a broad range of chemokine receptors and has been shown to inhibit signaling and migration induced by other chemokines (25), although both vMIP-I and II have been shown to act as agonists. Promiscuity of binding within a family of receptors is not unusual for chemokines, however binding between families is exceptional. vMIP-II may act as a selective Th2 chemoattractant by agonism via CCR8 molecules (26). The function of the third HHV8 chemokine homologue, BCK (23,24), has not been studied to date. HHV6 also encodes a putative chemokine homologue on U83 (27), although the homology is weak to known viral and cellular chemokines.

Why chemokine receptors are so well-represented within herpesvirus sequences in comparison to other regulators of immune response is unclear. It is possible that their intimate involvement with lymphocyte migration and adhesion presents a critical determinant for the *Herpesviridae*, which as a group are strongly cell associated and in most cases are partly or entirely resident in leukocytes during their life-cycle. If one may conclude that the herpesviruses as a group use chemokines and their receptors as important modulators of infection, the predominance of β-chemokine receptor homologues in the *betaherpesvirinae*, the presence of an α-chemokine receptor and β-chemokine homologues in the *gammaherpesvirinae* and the absence of either in the *alphaherpesvirinae* may be important determinants for the infection pattern typical for these groups. The existence of viral chemokine and chemokine receptor homologues with unusually broad binding capacity such as vMIP-II and US28 may also fulfil specific roles in infection.

Cytokine Homologues

The cytokine- and the chemokine- network act together to regulate immune responses towards pathogens or tumor cells. The cytokines can be divided into two groups according to the type of T

Table 3. Chemokine homologs

Viral Homolog	Familial Resemblance	Closest Cellular Protein	BLAST Identity	Accession Number
HHV8 vMIP-I / K6	CC chemokine	MIP-1alpha	43%	U75698
HHV8 vMIP-II / K4	CC chemokine	MIP-1alpha	52%	U75698
HHV8 BCK	CC chemokine	Eotaxin	36%	U83351

helper cell by which they are secreted. T lymphocytes of the Th1 type secrete the cytokines IL-2, IL-12, IFNγ and TNFβ whereas the Th2 type lymphocytes secrete the cytokines IL-4, IL-5, IL-6, IL-10 and IL-13. There is crossregulation between the two types of lymphocytes as the Th1-cytokines suppress the secretion of Th2-cytokines while stimulating their own secretion and vice versa (28). Thus the cytokine network can be easily influenced by differential secretion of certain cytokines to alter immune responses. The Th1 cells are responsible for the development of the cell-mediated immune response critical for the removal of intracellular pathogens such as certain bacteria and viruses. The Th2 cells are responsible for the development of high levels of IgG1, IgA and IgE production by B cells and for the activation of effector cells such as eosinophils. This response is critical for the successful removal of certain parasites and for controlling the cell-mediated response (29). Members of the family *Herpesviridae* take advantage of the immunoregulatory properties of cytokines in order to escape the immune system (Table 4).

The human herpes virus-4 (HHV4), also known as Epstein-Barr-Virus (EBV), a causative agent for infectious mononucleosis encodes a homologue of the cellular cytokine IL-10 (30). This viral IL-10 (vIL-10) shows 81% homology to human IL-10 and is expressed in the late phase of lytic infection (31). IL-10 has been described to have inhibitory effects on the immune response as it suppresses self destruction in autoimmune diseases (32), inhibits graft rejection (31) and is a mediator of tumor immune escape (33). Additionally it suppresses expression of MHC molecules (34) as well as costimulatory molecules on professional-antigen presenting cells (APC) (35). Recently vIL-10 has been identified as the mediator of tolerance induction towards B7-dependent antigens (35). On the other hand IL-10 stimulates B cell proliferation (31). The properties of vIL-10 have been demonstrated to alter immune responses in favor of the virus and ease virus infection during primary infection as well as virus reactivation. As IL-10 stimulates B cell proliferation it enhances the number of possible targets for virus infection. This is especially important during primary infection with EBV (31). Furthermore vIL10 suppresses the T cell mediated immune response by directly blocking activation of T cells (36). Not only HHV4 but also equine herpesvirus 2 (EHV2) codes for an IL-10 homologue which shows 76% amino acid homology to human IL10 (37,38).

There is another example of a gammaherpesvirus-encoded cytokine homologue which enhances proliferation of the cells targeted by the virus. Human herpes virus-8 (HHV-8) which is associated with Karposi's sarcoma (KS), body-cavity-based B cell lymphomas (BCBL) and multicentric Castleman's disease codes for a gene homologous to the human IL-6 gene. IL-6 originally termed B cell growth factor, is an essential cytokine required for growth and differentiation of B lymphocytes and B cell derived lymphomas, and is also secreted by different tumor cells and cell lines (39). HHV8 orf K2 encodes vIL6 which possesses 25% identity with human IL6 (Table 4). The highest degree of conservation is in the IL-6 domain known to bind the receptor (40). Binding of the receptor by vIL-6 has been described to enhance growth of IL-6-dependent cell lines (40). A possible role of vIL-6 in KS pathogenesis is supported by the finding that KS infected spindle cells express the high affinity IL-6 receptor *in vivo* (41). vIL-6 is found in KS lesions to a limited extend, while it is clearly expressed in lymphoproliferative disorders like BCBL (42). These findings support the hypothesis that vIL-6 contributes to the pathogenesis of HHV-8 associated diseases.

Not only cytokines are encoded by herpesviruses but also regulatory factors of cytokine genes. HHV-8 encodes a gene on K9 which shows homology to the family of interferon (IFN) regulatory factors (IRF). IRFs are DNA binding proteins which regulate the

Table 4. Cytokine homologs

Viral Homolog	Familial Resemblance	Closest Cellular Protein	BLAST Identity	Accession Number
EBV vIL 10	IL10	IL10	81%	P03180
HHV8 vIL6	IL6	IL6	25%	2246551
HHV8 K9	IFN regulatory factor	ICSBP	20%	2246536

expression of IFN by stimulating (IRF-1) or blocking transactivation (IRF-2) of IFN gene transcription (43). The K9 gene product vIRF has only a low overall amino acid identity to human IRF family members, the best-fit given by an advanced gapped BLAST search being 20% identity with IFN consensus sequence binding protein (ICSBP) (Table 4). However the greatest homology is found in the N-terminal region of the protein (70%) which forms the DNA binding motif (44). vIRF binds the IRF consensus element but does not transactivate transcription thus inhibiting IFN production (45).

Apoptosis-Regulating Homologues

Apoptosis or programmed cell death is one of the principle mechanisms the immune system uses to eliminate virally-infected cells, and there are many examples of viral mechanisms preventing apoptosis. The use of homologues of cellular genes to block apoptosis is characteristic of the *gammaherpesvirinae* and is not generally shared by other herpesviruses. Apoptosis generally can be triggered by receptors such as CD95, by granzymes, by radiation, serum starvation or ceramides (46). Once initiated a complicated cascade of signalling events leads to fragmentation of DNA, membrane blebbing and death of apoptotic cells. The continued viability of cells is dependent on a constant balance between pro-and anti-apoptotic signals. All viral homologues of cellular apoptosis-related proteins identified to date inhibit the apoptosis induced by viral infection and subsequent immune assault. Within the *gammaherpesvirinae* both apoptosis via the mitochondria or via death-receptors can be blocked by viral homologues of the cellular genes bcl-2 (B cell lymphoma/leukemia 2) and FLIP (FLICE (Fas-associated death-domain-like IL1β-converting enzyme) inhibitory protein) (Table 5).

The family of cellular bcl-2 proteins contains over 10 members (47,48), homology within the family being found in four bcl-2 homology domains (BH1–4). Bcl-2 is an anti-apoptotic protein that is principally responsible for inhibition of mitochondrial-triggered apoptosis. Viral bcl-2 homologues are conserved within the *gammaherpesvirinae*: BHRF1 from EBV and the product of orf 16 of HHV8 show homology to human bcl-2, although they are slightly shorter. Both viral homologues have anti-apoptotic functions similar to bcl-2: BHRF1 blocks apoptosis induced either by mitochondrial (49,50) or death-receptor (51,52) pathways, and HHV8 orf 16 similarly blocks apoptosis (53,54). However, functionally both differ in certain respects to the cellular bcl-2. BHRF1 can enhance cell proliferation (55) whereas cellular bcl-2 is inhibitory (56,57). Mutation of a tyrosine residue within the BH4 region (58), or deletion (59,60) of regions between BH3 and BH4 do not reduce the anti-apoptotic function of bcl-2 but do remove the block to cell proliferation. Furthermore cellular bcl-2 can be converted to an apoptosis-inducing agent by BH3-dependent cleavage by caspases (61). Concordant with the functional differences between cellular and viral bcl-2 neither BHRF1 nor orf 16 possess this region, and homology between viral bcl-2 and cellular bcl-2 is weakest in the BH3 and BH4 regions. The difference between viral and cellular bcl-2 therefore probably represent a functional pro-viral modification as opposed to random genetic drift. Herpes virus saimiri (HVS) ECLF2 (22) and murine γ 68 M11 (20) both also bear homologues to cellular bcl-2, demonstrating conservation within the *gammaherpesvirinae*.

Apoptosis can be induced by signaling via death receptors which are characterized by intracellular domains called death-effector domains (DED). These interact inside the cell with other DED-containing molecules such as FLICE which leads to the formation of the death inducing signaling complex

Table 5. Apoptosis-regulating homologs

Viral Homolog	Familial Resemblance	Closest Cellular Protein	BLAST Identity	Accession Number
EBV BHRF1	bcl	bcl-2	28%	A22899
HHV8 vbcl-2	bcl	MCL1	28%	U75698
HHV8 vFLIP	FLIP	FLIP	29%	U93872
HSV1 ICP34.5	GADD34	GADD34	32%	P08353

(DISC). Thereafter a cascade of caspases carries the signal to the effector mechanisms that cause the characteristic changes of apoptosis. HHV8 encodes on orfK13 a homologue of an apoptosis-related protein that inhibits FLICE, and is therefore called FLICE inhibitory protein (FLIP). Identified originally by two tandemly arranged regions approximately 25% homologous to cellular DEDs, the cognisance of viral FLIP (62) lead to the search for and identification of the cellular homologue (63). Both cellular and viral FLIP lack caspase activity and inhibit apoptosis by binding to death effector domain (DED) containing proteins such as Fas associated death domain protein (FADD) and FLICE, and thereby prevent caspase-8 recruitment. Thus vFLIP can block apoptogenic signalling induced by a variety of DED-containing death receptors (62). The equine herpesvirus protein E8 is homologous to vFLIP and can similarly block apoptosis (64,65).

One alphaherpesvirus, herpes simplex virus type 1 (HSV1), encodes a homologue of an anti-apoptotic gene. HSV1 ICP34.5 has sequence homology to two related cellular genes, GADD34 (Growth Arrest and DNA Damage inducible) and MyD166 (Myeloid Differentiation primary response gene 166) (66,67,68). The GADD family of proteins are induced as their name implies by DNA damage, and are closely linked with cell cycle arrest and apoptosis. ICP34.5 prevents the shutdown of protein synthesis that is normally caused by viral infection and can inhibit cell death after infection of certain cell types, particularly neuronal cell lines (67). This effect is dependent on the carboxy-terminal domain which has strong homology to the corresponding domain of GADD34 and MyD166 (69). ICP35.5 redirects protein phosphatase 1α to dephosphorylate eukaryotic translation initiation factor 2 (eIF-2) α subunit in order to maintain protein synthesis (69,70). In this fashion HSV1 ameliorates the effects of apoptotic signaling by using a homologue of a cellular protein.

Major Histocompatibility Complex (MHC) Homologues

The best characterized example is HCMV UL18 which is homologous with human MHC class I (71) (Table 6), having a sequence implying three extracellular domains. UL18 demonstrates functional similarity as well as sequence homology to MHC class I as it can both complex with β 2-microglobulin (72) and also present peptides (73). A homologous gene named M144 is found on MCMV (16). It has been proposed that both UL18 and M133 can inhibit natural killer (NK) cell-mediated attack (74,75) caused by the reduction of endogenous MHC class I expression on infected cells according to the missing self hypothesis. The missing self hypothesis of Kärre (76) proposes that MHC class I molecules are responsible for negative signaling to NK cells, preventing cytotoxic attack, and that if MHC class I molecules are lost then the cell is rendered vulnerable to NK cell attack. Subsequent research identified a novel immunoglobulin superfamily receptor termed leukocyte immunoglobulin-like receptor (LIR-1) that binds to UL18 (77). LIR-1 is related to killer cell inhibitory receptors (KIR), but is found predominantly on monocytic and B lymphoid cell types. It has also been demonstrated that cell lines transfected with UL18 suffer enhanced cytotoxicity and are not protected from NK-mediated cytotoxicity by interactions with KIR (78). Therefore the true function of UL18 remains unclear, despite its proven homology to cellular MHC class I molecules.

Complement-Regulating Protein Homologues

The complement system is an ancient component of the innate immune response to infection, and several herpesviral proteins show homology to proteins involved in the complement pathway. HSV1 glycoprotein gC-1 can bind C3b complement protein and

Table 6. Other homologs

Group	Viral Homolog	Familial Resemblance	Closest Cellular Protein	BLAST Identity	Accession Number
MHC class I homolog	HCMV UL18	HLA	HLA-A	27%	X17403
CCP homolog	HHV8 CCPH	CCP	CD46	34%	U93872

moderate complement attack, but the regions with strongest homology to cellular proteins are not required for this interaction (79). HHV8 encodes a gene on orf 4 which has homology to cellular complement control protein (CCP) (44) (Table 6), which regulate complement-mediated attack on the plasma membrane of cells. HVS also encodes a CCP homologue which has been shown to protect infected cells from complement-mediated attack (80,81). Complement is particularly important in the inactivation of virions, and homologues that act against complement may be more important to protect the virion than the infected cell.

Fc-Receptor Homologues

Many cells of the immune system express Fc receptors, molecules that act as receptors of the Fc portion of antibodies and bind them to the cellular surface. Herpesviruses also express Fc receptors which often bear homology to their cellular counterparts. HSV1 gE is a viral Fc receptor with a low affinity for IgG which is complemented by viral gI to become a high affinity Fc receptor (82). According to the bipolar bridging model the Fc domain of anti-HSV IgG that is in contact with a viral antigen is bound by viral Fc receptors, preventing fixation of complement and subsequent lysis of the infected cell. gE bears a region with homology to cellular Fc receptors that is both important for Fc binding (83) and for immune evasion (84). Other herpesvirus-encoded Fc receptors such as varicella-zoster virus (VZV) gpI do not, however, have significant homology to cellular Fc receptors (85). The Fc receptor may also be important for binding to other immunoglobulin superfamily proteins, as the Fc receptor encoded by MCMV (86) confers replicative advantage independent of antibody (87).

Ig-Superfamily Proteins

Several of the herpesviruses bear proteins with homology to immunoglobulin superfamily proteins, indicating homology to cellular proteins. However the immunoglobulin family is diverse in function and so although one can predict that these proteins derive from cellular proteins, their direct function and homologue is obscure. Within this grouping we place: HHV7 U20, HHV6 U20 (6) which has homology to IgE chain C (27), HHV7 U84 and HHV6 U85, and HCMV UL122 (6). HHV8 K14 (44) and HHV6 U85 are homologues of neural cell adhesion molecule (N-CAM) family transmembrane proteins. N-CAMs are normally found on NK cells or neurons and are important in both immune regulation and neuronal development.

Other Homologues

There are naturally a number of other viral proteins bearing strong homology to cellular proteins that are, however, of less interest immunologically. Within this group are many of the genes essential for viral replication, such as DNA polymerase, where structure is strongly conserved due to the constraints of host metabolism. Herpesviral DNA polymerases are very similar to cellular DNA polymerase α, for example six regions in the DNA polymerase of HSV, HCMV and EBV show similarity with human DNA polymerase and are similarly arranged (88). HHV8 orf 72 codes for a viral homologue of cylin D which shows 31% amino acid identity with cellular cyclin D2 and 25% identity with D1. The viral homologue associates predominantly with cyclin dependent kinase (cdk) 6 (89), cdk6 activity being predominantly found in lymphoid cells. VZV gene 13 shows close homology to thymidylate synthetase (90). Most herpesviruses except HHV6 and HCMV encode homologues of cellular thymidine kinases which differ functionally in their substrate specificities (91). HCMV UL97 encodes a phosphotransferase which shows homology to protein kinases (92), as do other herpesviral proteins such as HSV1 US3 (93).

Molecular Mimicy and the Danger of Autoimmunity

The immunological significance of viral homologues of cellular genes goes beyond replication or modification of the host immune response in order to facilitate infection. A variety of studies have demonstrated a link between autoimmune disease and virally-encoded peptides or molecular mimics that are homologous with endogenous peptides. The thesis of molecular mimicry was first proposed by Oldstone et al. (94). This states that virus encoded mimetic

peptides derived from homologues of cellular proteins will be presented to the immune system in stimulatory ways, for example under conditions of inflammation, and tolerance to the corresponding endogenous protein may therefore be overcome. The incorporation of cellular genes into viral genomes that are present for the lifetime of the host, as is the case with herpesviruses, therefore elevates the risk of a breakdown in self-tolerance and may be an initiator or contributory factor to autoimmune diseases.

Therapeutic Implications of Herpesvirus Homologues of Cellular Proteins

Although viral homologues are often similar in function to the cellular protein, the precise mechanism has frequently been altered during the co-evolution of virus and host. Viral homologues may therefore be used as targets of therapy, utilising the change in protein function to selectively eliminate infected cells. The best examples of such an approach are the drugs acyclovir and gancyclovir, guanosine anologs which are preferentially phosphorylated into their active form by viral thymidine kinase, cellular thymidine kinase having little or no activity. Other well-conserved homologues such as the UL33 group may prove to be suitable targets for therapeutic agents. Alteration of function between viral homologue and cellular protein can be further exploited to treat disease, for example utilising the broad binding activity of HHV8 chemokine homologues to block HIV infection of T cells (24).

Concluding Remarks

We now possess the partial or complete sequences of major pathogenic large DNA viruses representing a valuable repository of information on the mechanics of the immune system. In particular fascinating are the *Herpesviridae*, an ancient virus family highly adapted to its host with very large genomes encoding hundred of proteins of which many are involved in an stunning variety of different mechanisms of viral immune evasion. The existence of a viral homologue of a cellular protein where the function of both is unknown per se suggests that the protein may play a role in the immune system. This has been shown by vFLIP: the discovery of DED domains was a clue to the discovery vFLIP which in turn led back to cellular FLIP. This story illuminates the significance of herpesviruses as research tools beyond their technical utility as transfection systems or clinical significance as vectors of disease. The study of the immunomodulatory proteins could reveal new aspects of viral pathogenesis and help to tailor vaccines and to treat infectious diseases. Moreover this knowledge could lead to the development of novel immunotherapeutic strategies in transplantation medicine and treatment of virus-associated cancer. Powerful immunological weapons indeed have been created in the struggle between Man and Virus. The list is by far not complete; virologists and immunologists have yet to unravel more exciting examples in the future.

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Iridovirus Homologues of Cellular Genes—Implications for the Molecular Evolution of Large DNA Viruses

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Abstract. Iridoviruses belong to the group of large cytoplasmic deoxyriboviruses and infect either insects or vertebrates. In analogy to other large DNA viruses of eucaryotes it was found that iridoviruses encode a number of cellular protein homologues. The majority of these proteins represent orthologues of cellular enzymes involved in transcription, replication, and nucleotide metabolism. Others may have the potential to interfere with cell cycle regulation or immune defence mechanisms of the host. This raises the question about the phylogenetic origin of the corresponding viral genes. During the evolution of large cytoplasmic DNA viruses such as iridoviruses, poxviruses, and *African swine fever virus* the acquirement of cellular genes appears to be a crucial event. Each member of this group of viruses encodes a DNA polymerase, two subunits of the DNA-dependent RNA polymerase, and two subunits of the ribonucleotide reductase. It is important to note that all of these viral proteins show a high level of multidomain structure conservation as compared to their cellular orthologues. As a consequence the large cytoplasmic DNA viruses have the ability to replicate independently of the cellular nucleus in the cytoplasm of the infected cell. Assuming a common cellular origin of viral DNA polymerase genes the corresponding amino acid sequences were chosen to construct a phylogenetic tree showing the relatedness among large DNA viruses of eucaryotes.

Key words: *Iridoviridae*, DNA viruses, molecular evolution, cellular genes, computer analysis, sequence alignment

Introduction

The complex genomes of cells and the comparatively small genomes of viruses are products of a long and still ongoing coevolution process (1). The fitness of a particular virus in relation to its host cell is determined by the occurrence of mutations and genetic rearrangements that result in improvements and adaptation on the protein level. Since viruses have very short generation times and produce large amounts of progeny they have the potential to evolve much faster than any living organism. In addition, many viruses use particularly error prone nucleic acid polymerases for their replication (2).

As a common feature large DNA viruses encode functional homologues of cellular proteins. This raises the question about the phylogenetic origin of the corresponding viral genes. One possibility is that viruses and cells have evolved analogous three-dimensional protein structures that are able to catalyze the same biochemical reaction. Generally speaking, such a convergent development would result in more or less unique proteins that contain structurally related functional domains. Another possibility is that viruses have acquired cellular genes by recombination mechanisms and that these genes diverged over time in order to suit the virus-specific requirements. In this particular case one would not only expect significant amino acid sequence homology that is confined to isolated functional motifs, but also a colinear overall organization of the individual conserved domains within the primary structure of the orthologous viral and cellular proteins. The uptake of cellular genes into the viral genome may be of significant advantage for

large DNA viruses that replicate in the nucleus of the host cell such as herpesviruses and baculoviruses. But even more importantly, the acquirement of foreign genes appears to be a crucial event in the evolution of large cytoplasmic DNA viruses such as iridoviruses, poxviruses, and *African swine fever virus*. By encoding their own orthologues of cellular enzymes involved in transcription, replication, and nucleotide metabolism these viruses have gained independence of the cellular nucleus.

Iridovirus-Encoded Homologues of Cellular Genes

Iridoviruses are large icosahedral cytoplasmic deoxyriboviruses that can be subdivided into at least four genera infecting either insects or cold-blooded vertebrates (3). Some members of the family *Iridoviridae* have attracted much attention because of their ecological and economic impact. These include lymphocystis disease virus (LCDV), which naturally occurs in a large number of different fish species world-wide (4), and *Chilo* iridescent virus (CIV) causing lethal disease in important pest and vector insect species (5,6). The genome of iridoviruses, typically ranging from 100 to 210 kbp in size, is represented by a single linear double-stranded DNA molecule that is circularly permuted and terminally redundant (3). During the last years there have been reports of a number of new iridovirus isolates from all over the world (7–9). However, the available nucleotide sequence information is still very limited and in most cases it is restricted to the coding region of the viral major capsid protein gene. Until today, LCDV and CIV are the only two iridovirus species for which substantial portions of the genomic primary structure have been determined (10–12). The knowledge of the genome structure, gene content, and coding strategy of these two representative iridovirus species infecting vertebrates on one hand and insects on the other allow the analysis of the evolutionary relatedness of gene orthologues among iridoviruses, other large DNA viruses, and their eucaryotic hosts. Known iridoviral orthologues of cellular genes that show a high level of multidomain structure conservation are summarized in Table 1. Each of the twelve iridovirus proteins belongs to a different well-characterized eucaryotic protein family. It is obvious that the majority of these proteins is involved in the nucleic acid metabolism.

Like other cytoplasmic DNA viruses the irido-

Table 1. Known viral orthologues of cellular genes encoded by members of the family *Iridoviridae* and other large DNA viruses infecting eucaryotes

Protein	IV	Gene	Acc. No.	Other Large DNA Viruses Infecting Eucaryotes
DNA polymerase	CIV	A031L	AF083915	*Ascoviridae, Asfarviridae, Baculoviridae,*
	LCDV	135R	L63545	*Herpesviridae, Phycodnaviridae, Poxviridae*
	RSIV	n.a.	AB007366	
ribonucleotide reductase large subunit	CIV	028L	AF003534	*Asfarviridae, Baculoviridae, Herpesviridae,*
	LCDV	176L	L63545	*Phycodnaviridae, Poxviridae*
ribonucleotide reductase small subunit	LCDV	027R	L63545	*Asfarviridae, Baculoviridae, Herpesviridae, Phycodnaviridae, Poxviridae*
DNA-dependent RNA polymerase II subunit 1	CIV	097R	AF003534	*Asfarviridae, Poxviridae*
	LCDV	016L	L63545	
DNA-dependent RNA polymerase II subunit 2	LCDV	025L	L63545	*Asfarviridae, Poxviridae*
DNA topoisomerase II	CIV	A039L	AF083915	*Asfarviridae, Phycodnaviridae*
thymidylate synthase	CIV	TYSY	AF059506	*Herpesviridae*
3-β-hydroxysteroid dehydrogenase	LCDV	153L	L63545	*Poxviridae*
cathepsin B-like cysteine proteinase	LCDV	043R	L63545	*Baculoviridae*
SNF2-like DNA helicase	CIV	095L	AF003534	*Baculoviridae*
PIF1-like DNA helicase	CIV	A027L	AF083915	none
cytoplasmic 5′-3′ exoribonuclease	CIV	A019L	AF083915	none

Note: Included are only those protein families for which a high level of multidomain structure conservation is evident. Abbreviations: IV = iridovirus species, CIV = *Chilo iridescent virus*, LCDV = *Lymphocystis disease virus*, RSIV = *Red sea bream iridovirus*, Acc. No. = GenBank Accession Number, n.a. = name not available.

viruses encode a DNA polymerase (10,12) as well as the two subunits of a DNA-dependent RNA polymerase (10,11,13,14), which are required for virus replication and transcription. In addition, it was found that vertebrate iridoviruses encode a C-5 cytosine-specific DNA methylase (15,16). Genes encoding a DNA topoisomerase type II and at least two different DNA helicases were identified in the genome of CIV (11,12,17). Iridovirus-encoded enzymes that interfere with the nucleic acid metabolism of the host cell include the two subunits of the ribonucleotide reductase, a thymidine kinase (10,11), a thymidylate synthase (18), and a cytoplasmic 5′-3′ exoribonuclease (12). Although both, CIV and LCDV, encode several putative protein kinases, the only significant iridovirus homologue of cellular protein-modifying enzymes is the LCDV-1 cathepsin B-like cysteine proteinase (10).

Iridoviruses also encode homologues of cellular proteins that have the potential to directly interfere with the cell cycle or the immune system of the host organism. These include inhibitors of apoptosis (11), an insulin-like growth factor, a 3-β-hydroxysteroid dehydrogenase, and a soluble tumor necrosis factor receptor homologue (10). However, the actual function of these proteins *in vivo* remains to be elucidated.

Phylogenetic Relatedness Among Large DNA Viruses of Eucaryotes

A major obstacle for a DNA virus infecting a eucaryotic cell is the limited availability of free deoxyribonucleotides, which are required for viral DNA replication. Interestingly, small and large DNA viruses have evolved different ways to overcome this obstacle. For example, members of the *Adeno-* and *Papovaviridae* induce cellular S-phase progression to provide the substrates for viral DNA synthesis (19). In contrast, large DNA viruses encode enzymes like for example the ribonucleotide reductase, which catalyses the reductive synthesis of deoxyribonucleotides from the corresponding ribonucleotides. As a consequence the host cell nucleotide metabolism is permanently shifted towards DNA synthesis and the viral DNA polymerase can produce virus progeny independently from the cellular S-phase.

Among the different iridovirus-encoded homologues of cellular proteins the DNA polymerase and the two subunits of the ribonucleotide reductase are the only proteins that are common to all families of large DNA viruses of eucaryotes examined to date (see Table 1). For an unknown reason some large DNA virus species do not encode a ribonucleotide reductase (e.g. betaherpesviruses and some poxviruses). However, at least in the case of *Fowlpox virus* there is some evidence that the gene encoding the large subunit of the ribonucleotide reductase has been replaced by another gene during evolution (20).

The DNA polymerase is among the best-studied proteins of large DNA viruses and it has proven to be suitable for the determination of phylogenetic relationships (21). Based on the multiple amino acid sequence alignment of viral DNA polymerases a phylogenetic tree was constructed showing the relatedness among large DNA viruses of eucaryotes (Fig. 1). It is obvious that the clustering of different family and subfamily members is in perfect agreement with the current virus taxonomy (22). Interestingly, the phylogenetic tree based on the viral DNA polymerases does not show any clustering regarding common host ranges of the individual virus species. For example there is no predominant relationship among DNA polymerases of insect viruses belonging to the *Iridoviridae*, *Ascoviridae*, *Baculoviridae*, and *Poxviridae* families. This finding suggests that the uptake of a cellular DNA polymerase gene into the genome of possible ancestors of today's large DNA viruses is not a recent event. Another argument against the hypothesis of a convergent evolution of viral DNA polymerases is the fact that the relationship of different virus families based on the viral DNA polymerase does not reflect common replication mechanisms. For example, poxviruses, phycodnaviruses, and *African swine fever virus* possess a linear double-stranded DNA genome with inverted terminal repeats and covalently closed hairpin ends (22). However, the DNA polymerase of phycodnaviruses shows highest homologies to DNA polymerases of iridoviruses and herpesviruses, which have a completely different genome structure and replication mechanism. Therefore the common functionality of DNA polymerases of poxviruses, phycodnaviruses, and *African swine fever virus* is not likely to be the result of a convergent evolution.

In contrast to the DNA polymerase there is evidence that the genes encoding the ribonucleotide reductase may have been acquired by different virus species independently. Firstly, the phylogenetic

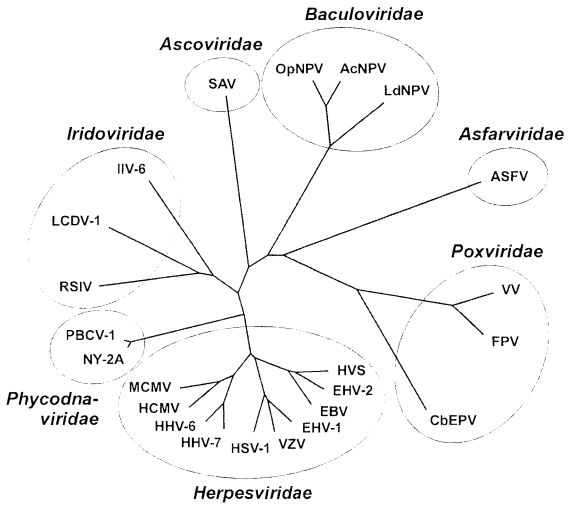

Fig. 1. Phylogenetic tree of large DNA viruses infecting eucaryotes. The unrooted tree is based on a protein cluster alignment of the virus-encoded DNA polymerases and was generated using the PHYLIP package (version 3.5c, distributed by J. Felsenstein, Department of Genetics, University of Washington, Seattle). Branch lengths represent relative phylogenetic distances according to maximum likelihood estimates based on the Dayhoff PAM matrix. Clusters of related virus family members are indicated. The following virus species are included in the cluster alignment (GenBank/EMBL/SwissProt accession numbers are listed in brackets): AcNPV = *Autographa californica nucleopolyhedrovirus* (P18131), ASFV = *African swine fever virus* (P42489), CbEPV = *Choristoneura biennis entomopoxvirus* (P30319), EBV = *Epstein-Barr virus* (P03198), EHV-1 = *Equine herpesvirus 1* (P28858), EHV-2 = *Equine herpesvirus 2* (P52367), FPV = *Fowlpox virus* (P21402), HCMV = *Human cytomegalovirus* (P08546), HHV-6 = *Human herpesvirus 6* (P28857), HHV-7 = *Human herpesvirus 7* (P52342), HSV-1 = *Herpes simplex virus 1* (P04293), HVS = *Herpesvirus saimiri* (P24907), IIV-6 = *Invertebrate iridescent virus 6* (*Chilo iridescent virus*, AF083915), LCDV-1 = *Lymphocystis disease virus 1* (L63545), LdNPV = *Lymantria dispar nucleopolyhedrovirus* (P30318), MCMV = *Mouse cytomegalovirus* (P27172), NY-2A = *Paramecium bursaria Chlorella virus NY-2A* (M86837), OpNPV = *Orgyia pseudotsugata nucleopolyhedrovirus* (Q83948), PBCV-1 = *Paramecium bursaria Chlorella virus 1* (U42580), RSIV = *Red sea bream iridovirus* (AB007366), SAV = *Spodoptera ascovirus* (U35732), VV = *Vaccinia virus* (P06856), VZV = *Varicella-zoster virus* (P09252).

analysis of the amino acid sequences of the viral ribonucleotide reductase subunits is not always in agreement with the current virus taxonomy and in some cases reflects common host ranges rather than family membership (data not shown). Secondly, the ribonucleotide reductase appears to be a non-essential gene product since some large DNA viruses do not encode this enzyme (e.g. betaherpesviruses) and some might have lost the corresponding gene during evolution (e.g. *Fowlpox virus*, 20).

In conclusion it appears that some genes of large DNA viruses encoding enzymes of the nucleic acid metabolism indeed originated from cellular ancestors. However, the question remains about the fate of the introns that were present in the original cellular genes. One possibility is that the acquirement of cellular genetic information occurred through retrotransposition, which implies that a DNA copy of a spliced cellular mRNA integrates into the viral DNA genome. There is evidence that retroviruses can integrate into the genome of herpesviruses suggesting a potentially important role of such a mechanism in herpesvirus evolution (23). Another possibility is the direct recombination of viral DNA with the cellular genome followed by subsequent deletion of non-coding sequences. This may be an explanation for the presence of small introns in the DNA polymerase genes of phycodnaviruses (24). In fact, the actual mechanisms of such recombination events between viral and cellular genomes are not well studied. However, it seems that genetic interchange should be of evolutionary benefit for both interactive partners—the viruses and their eucaryotic hosts.

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PART C:

Poxvirus Gene Homologues of Cellular Genes

Virus Genes 21:1/2, 85–95, 2000

Sequence and Functional Analysis of a Homolog of Interleukin-10 Encoded by the Parapoxvirus Orf Virus

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Abstract. Orf virus is a large DNA virus and is the type species of the *Parapoxvirus* genus of the family Poxviridae. Orf virus infects the epithelium of sheep and goats and is transmissible to humans. Recently we discovered a gene in orf virus that encodes a polypeptide with remarkable homology to mammalian interleukin (IL-10) and viral encoded IL-10s of herpes viruses. The predicted polypeptide sequence shows high levels of amino acid identity to IL-10 of sheep (80%), cattle (75%), humans (67%) and mice (64%), as well as IL-10-like proteins of Epstein-Barr virus (63%) and equine herpes virus (67%). The C-terminal region, comprising two-thirds of the orf virus protein, is identical to ovine IL-10 which suggests that this gene has been captured from its host sheep during the evolution of orf virus. In contrast the N-terminal region shows little homology with cellular IL-10s and in this respect resembles other viral IL-10s. IL-10 is a pleiotrophic cytokine that can exert either immunostimulatory or immunosuppressive effects on many cell types. IL-10 is a potent anti-inflammatory cytokine with inhibitory effects on non-specific immunity in particular macrophage function and Th1 effector function. Our studies so far, indicate, that the functional activities of orf virus IL-10 are the same as ovine IL-10. Orf virus IL-10 stimulates mouse thymocyte proliferation and inhibits cytokine synthesis in lipopolysaccharide-activated ovine macrophages, peripheral blood monocytes and keratinocytes. Infection of sheep with an IL-10 deletion mutant of orf virus has shown that interferon-γ levels are higher in tissue infected with the mutant virus than the parent virus. The functional activities of IL-10 and our data on orf virus IL-10 suggest a role in immune evasion.

Key words: orf virus, poxvirus, interleukin-10, gene function, sequence homology, evolutionary relationships, genetics

Introduction

Poxviruses include some of the most virulent pathogens of humans and animals. Members of the poxvirus family generally produce acute cytolytic infections and the success of these viruses can be attributed to their ability to replicate in the presence of an active host immune response. The genomes of poxviruses contain between 140–400 genes, approximately half of which encode factors that are essential for the replication of the virus in the cytoplasm of the cell while the remaining genes encode factors that modulate the host defences and modify cell physiological processes (reviewed in ref. 1–4). Most of the virulence factors are directed at suppressing inflammation and the innate responses in particular the complement system, interferons, natural killer (NK) cell activity and apoptosis. Many of these virulence factors have homology to cellular genes, which suggests that poxviruses have captured genes from their host during their evolutionary development.

We have been studying the molecular biology of orf virus (ORFV) with the aim of identifying genes that encode factors that are involved in immune evasion. ORFV is the type species of the parapoxvirus group in the family Poxviridae (5). ORFV infects

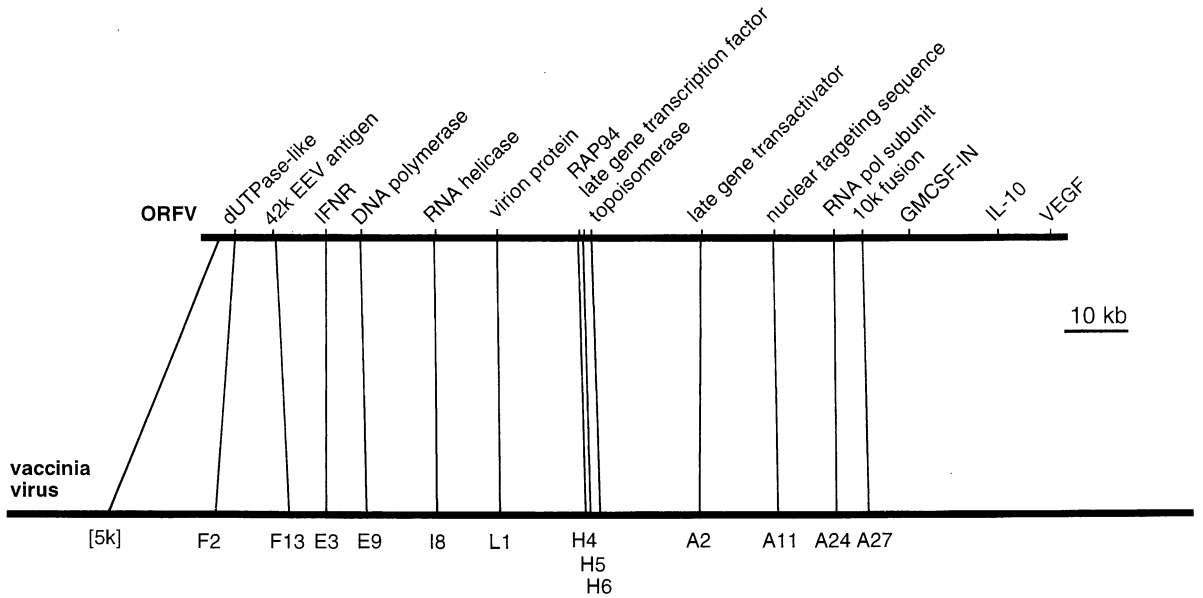

Fig. 1. Genetic alignment of the genomes of ORFV (NZ2 strain) and vaccinia virus (Copenhagen strain) (30). The heavy lines represent the genomes of each virus. A selection of ORFV genes that have homologs in vaccinia virus are shown (reviewed in refs. 7 and 21). The 5 k gene is bracketed to indicate that it is present in the WR but not the Copenhagen strain of vaccinia virus. The ends of each line drawn between the two genomes mark the location of each pair of homologous genes and the characteristics of these genes are shown. IFNR, GMCSF-In, IL-10 and VEGF mark genes encoding an interferon resistant element (24–25) an uncharacterized element associated with GM-CSF inhibitory activity (27), a homolog of IL-10 (28) and of vascular endothelial growth factor (26), respectively.

sheep and goats and is transmissible to humans. In sheep, the virus causes a disease called scabby mouth or contagious pustular dermatitis. The virus enters its host, usually through a break in the skin and replicates in keratinocytes of the epidermis (6, reviewed in ref. 7). Skin lesions begin as reddening and swelling around the sites of inoculation and these develop into small vesicles over 24 h. Several days after inoculation the vesicles develop a pustular appearance due to a large infiltration of polymorphonucleocytes. Adjacent lesions may coalesce as the disease progresses followed by scab formation over the surface of the lesion. The primary lesion normally resolves in 4–6 weeks (8) at which time the scab, containing virus particles, is shed. Virus replication is maximal between 5 and 7 days and is usually undetectable by day 14 (9–10). There is no evidence of systemic spread of ORFV (11), it rarely kills its host, but can cause an acute debilitating disease in cases where it infects the mouth parts or nares of the animal (8). Occasionally ORFV establishes a persistent infection in sheep, which is manifested as large tumor-like growths. In humans, a similar severe progressive disease has been reported in immunocompromised individuals (12–14). Severe reactions have also been recorded in apparently normal individuals (15), in cases where burns have become infected (16) and in cases of atopic dermatitis (17).

Analysis of the immune response to ORFV shows that an early neutrophil response within the first 48 h is followed by an accumulation of $\gamma\ \delta\ TCR^+$ T -cells, $CD4^+$ T cells, $CD8^+$ T-cells and B cells adjacent to and underlying the infected epidermal cells (10,18). $CD4^+$ T cells are numerically the predominant T cell. The most unusual feature of the ORFV lesion is a dense accumulation of MHC Class II^+ dendritic cells which lack the macrophage associated antigens, CD11b and CD11c (10,18). ORFV can repeatedly reinfect animals in spite of a vigorous inflammatory response and apparent specific host immune response. Reinfection lesions progress through the same clinical stages but generally are not proliferative, are smaller and resolve more rapidly usually within 2–3 weeks (6, reviewed in ref. 7, 19).

We have determined the DNA sequence of various parts of the 140 kb genome of ORFV and have thereby

shown that the genetic structure resembles vaccinia virus, the prototypal member of the orthopoxvirus genus (Fig. 1) (20–21). We have found that homologs of vaccinia virus structural proteins and proteins that are essential for replication are encoded within the central core of the ORFV genome and these genes are conserved in position, orientation and spacing (21). We have deduced that the difference in size of the genomes can be attributed to differences within the termini. In general, the termini of poxviruses encode factors that are involved in pathogenesis, virulence and host range and are non-essential for growth in cell culture (22–23). Within the termini of ORFV we have discovered a homolog of the vaccinia virus E3L interferon resistance gene (24–25), a homolog of vascular endothelial growth factor (VEGF) (26), a granulocyte macrophage colony stimulating factor (GM-CSF) inhibitory activity (27) and a homolog of interleukin (IL)-10 (28) (Fig. 1) which is the subject of this report.

Orf Virus Encodes IL-10

Sequence analysis of a 6.0 kb subfragment of *Kpn*I-E, strain NZ2, revealed an open reading frame with high identity to mammalian IL-10 and IL-10-like genes of members of the herpes virus family, Epstein-Barr virus (EBV) and Equine herpes virus 2 (EHV2) (28). A comparison of the predicted amino acid (aa) sequence of ORFV NZ2 IL-10 with those of other IL-10 sequences in the PIR database held by Genbank gave optimized scores which ranged from 745 to 594 (29). Ovine IL-10 showed 96% identity over 148 aa. The coding region of the ORFV NZ2 IL-10 gene is 561 nucleotides (nt) (Fig. 2). In keeping with other poxvirus genes ORFV NZ2 IL-10 does not contain introns unlike its cellular counterparts. The length of the predicted polypeptide of ORFV NZ2 IL-10 is 186 aa with a molecular mass of 21.74 kDa making it slightly larger than cellular and viral IL-10s.

Screening of other ORFV isolates by PCR amplification using primer sequences based on the ORFV NZ2 IL-10 gene suggests that all isolates carry this gene. We have identified a homolog of IL-10 in the New Zealand strain NZ 7 (ORFV NZ7) and our colleagues have identified this gene in a Scottish isolate, strain orf11 (C. McInnes pers comm) and a German isolate, strain D1701 (M. Buttner and A. Rhiza pers. comm.). Sequencing of ORFV NZ7 IL-10 revealed that there are few differences at the nt level between strains ORFV NZ2 and ORFV NZ7 (Fig. 2). Differences in the translated sequences were found near the N-terminus only. Restriction endonuclease analysis and Southern blotting showed that the two genes map to the same relative position in the viral genome.

Typical poxvirus early transcriptional regulatory sequences flank the ORFV NZ2 and ORFV NZ7 IL-10 genes (Fig. 2). An A + T-rich early promoter-like sequence is located upstream from the initiation codon while an early transcriptional termination sequence, TTTTTAT, is found downstream from the stop codon. The transcriptional regulatory sequences are highly conserved in each strain. The putative early promoter sequences are identical.

Northern analysis confirmed that ORFV NZ2 and ORFV NZ7 IL-10 are transcribed early. RNA was extracted from bovine testis cells that had been infected with ORFV in the presence of cycloheximide in order to block protein synthesis and subsequent transcription of intermediate and late genes. Northern blotting, using gene specific probes revealed transcripts of 0.8 kb and 2.3 kb in each strain (Fig. 3). We deduced from sequence data that mRNAs of approximately 760 nt would be expected to be transcribed from the IL-10 gene and the observed transcript of 0.8 kb corresponds well with this prediction. We deduced that the 2.3 kb transcript originated from an early gene upstream of IL-10. Sequence analysis of the upstream region revealed an ORF which encodes a polypeptide with ankyrin repeats and an early promoter sequence (unpublished data), however, this analysis did not reveal a transcription termination motif for this gene. A probe specific for the ankyrin-like gene detected a transcript of 2.3 kb. We concluded from this analysis that transcriptional read-through from the ankyrin-like gene was occurring and that this gene and the IL-10 gene share a common transcriptional termination sequence. Interestingly we have observed this phenomenon in other early genes encoded by ORFV, for example, a homolog of the vaccinia virus E3L gene shares a transcriptional termination sequence with an adjacent upstream gene (25) and early genes within ORFV NZ2 *Eco*RI-E share transcription termination sequences (unpublished). This phenomenon is not peculiar to ORFV and is also seen in vaccinia virus (30) and molluscum contagiosum virus (31). It seems likely that host gene regulatory sequences are replaced by

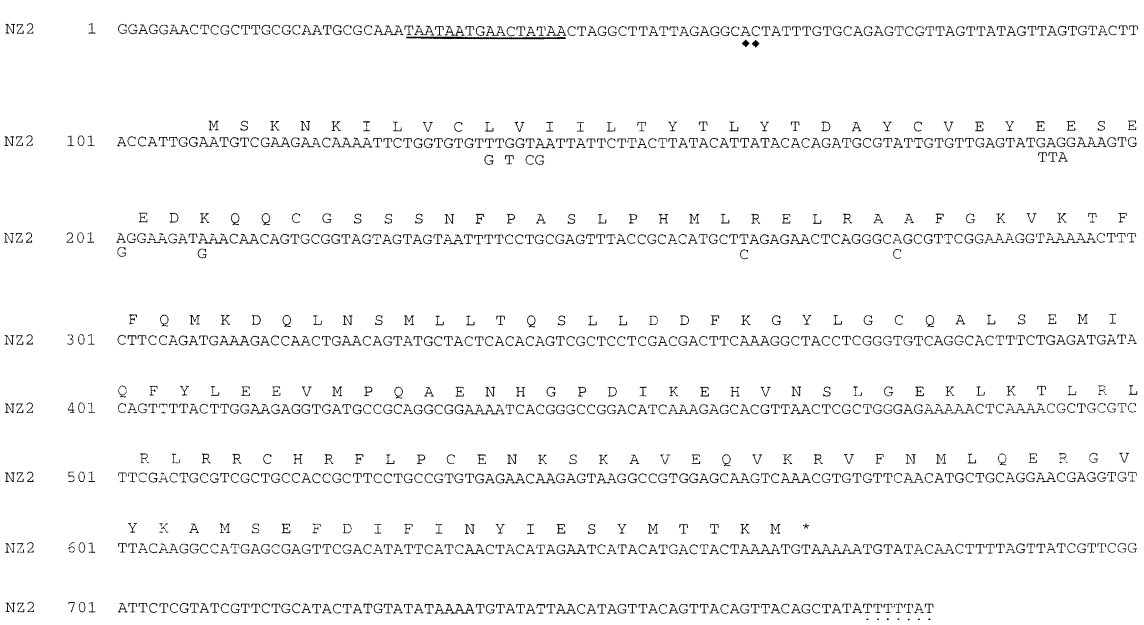

Fig. 2. Nucleotide sequence of the ORFV NZ2 IL-10-like gene. The order of amino acids, represented in one-letter code, was deduced from the nucleotide sequence. Differences in nucleotide sequence in ORFV NZ7-IL-10 are shown below the ORFV NZ2 sequence. Nucleotides in ORFV NZ2 which are not present in ORFV NZ7 are indicated by diamonds. The putative early promoter sequence is underlined and the early transcription termination sequence is shown by dots. (Figure reproduced from ref. 28.)

viral gene regulatory sequences following the capture of host genes and that the sharing of transcriptional control sequences may take place while these sequences are evolving. Alternatively, this may be a mechanism of regulating gene expression or a means by which the virus reduces its overall nucleic acid content without reducing its coding potential.

An alignment of the predicted aa sequences of the products of ORFV NZ2 IL-10 and ORFV NZ7 IL-10 with mammalian and herpes virus IL-10 sequences showed that the translated sequences of the IL-10-like genes are similar to sequences of other IL-10s (Fig. 4) (28). The homologies of the predicted polypeptide sequences of ORFV NZ2 IL-10 and ORFV NZ7 IL-10 with mammalian and viral IL-10-like proteins are as follows: ovine 80 and 79%, respectively; bovine 75 and 74%; human 67 and 67%; mouse 64 and 63%; EBV, 63 and 62%; EHV2, 67 and 66%. The identity of the IL-10-like genes is highest over the final two-thirds of the protein. This region of the protein is highly conserved across all mammalian species of IL-10 and herpes virus IL-10. Furthermore the ORFV IL-10 homologs are 98.6% identical with ovine IL-10 from aa 44 to the carboxy terminus. The relatedness of ORFV IL-10 to ovine IL-10 is less apparent at the DNA level (67% identity). This reflects differences in codon usage and the higher G + C content of ORFV genes in general.

Comparison of the N-terminal sequences (aa 1 to 42) of ORFV IL-10 polypeptides with their mammalian and viral counterparts reveals less homology. Part of this region is likely to comprise the secretory signal sequence of IL-10. The secretory signal sequences of mammalian IL-10s are between 18 and 19 aa while EHV2 IL-10 is predicted to be 25 aa. A domain in mammalian IL-10s which falls within the secretory signal has the consensus sequence ALLCCLVLLT/A. This consensus sequence is partly conserved in ORFV IL-10. Signal sequences are typically hydrophobic and a hydrophilicity plot (Kyte-Doolittle) revealed a strongly hydrophobic region of 18 aa at the N-terminus of ORFV IL-10. Furthermore a signal sequence analysis programme, Signal P (32), identified a putative signal sequence and predicted a cleavage site at 22–23 aa (TDA-YC).

The evolutionary state and phylogenetic relation-

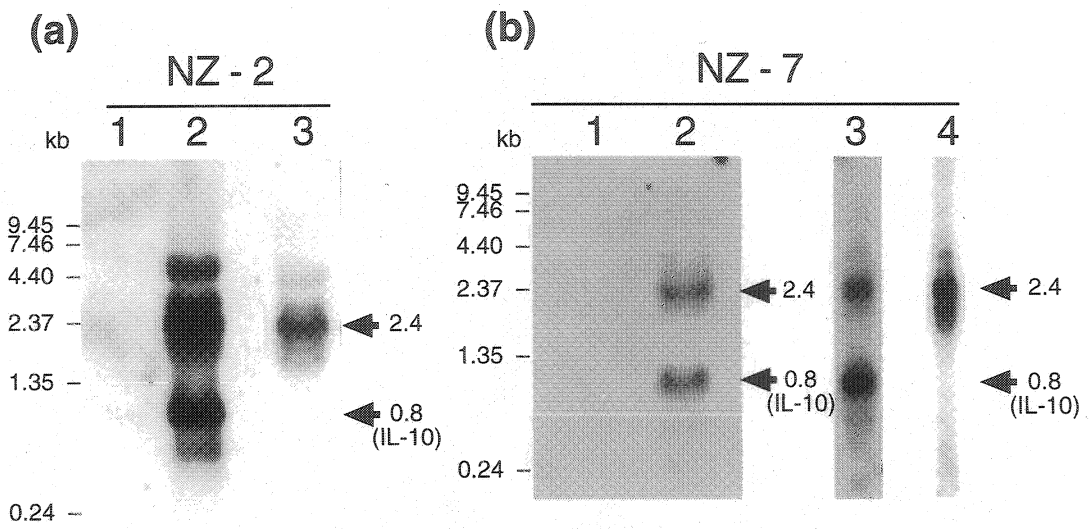

Fig. 3. Northern analysis of the IL-10 gene. Total RNA was isolated from bovine testis cells infected with either ORFV NZ2 or ORFV NZ7. Early RNA was isolated at 6 h postinfection from cells infected in the presence of cycloheximide. RNA was separated by electrophoresis in an agarose-formaldehyde gel and transferred to a nitrocellulose membrane. All membranes were hybridized with ^{32}P-labelled dsDNA probes. The IL-10 probe spanned the coding sequence of the IL-10 gene. The upstream probe spanned a region from the 5′ end of the putative IL-10 promoter (nt 4 [Fig. 2]) to a site 500 bp upstream of the IL-10 gene. (a) NZ-2. Lane 1, mock-infected RNA hybridized with IL-10 probe; lane 2, early RNA hybridized with IL-10 probe; lane 3, early RNA hybridized with upstream probe. (b) NZ-7. Lane 1, mock-infected RNA hybridized with IL-10 probe; lanes 2 and 3, early RNA hybridized with IL-10 probe; lane 4, early RNA hybridized with upstream probe. The blot shown in lanes 3 and 4 was prepared from a gel different to that shown in lanes 1 and 2. The position of the RNA markers are shown to the left. (Figure reproduced from ref. 28.)

ship of ORFV NZ2 IL-10 in comparison to other viral and cellular IL-10s were investigated using methods developed for constructing phylogenetic trees (33–34). A dendrogram of the alignment of IL-10 proteins is shown in Fig. 5. The phylogenetic relationship shows that ORFV IL-10 separates from the branch with ruminant which suggests that ORFV has captured the IL-10 gene from sheep, although a further possibility is that ORFV has captured this gene from goats. We have not been able to make this comparison since the sequence of goat IL-10 is not known. It is of interest that EBV IL-10 closely resembles human IL-10 and it has been suggested that EHV2 IL-10 represents a processed cellular gene, possibly captured from the horse, which suggests that there are constraints by selective pressure for the viral IL-10-like proteins to resemble their eukaryotic counterparts. The mechanism by which these viruses have captured host genes is not known, but it has been proposed that the IL-10 genes have been acquired via a step involving IL-10 mRNA and reverse transcriptase provided by a retrovirus since cellular IL-10s contain introns whereas viral IL-10s do not.

We were interested to find how wide-spread the IL-10 gene is in the parapoxvirus genus since this may indicate its significance in pathogenesis and virulence in closely related epitheliotropic viruses and possible evolutionary relationships between members of the group. Members of this genus that we examined for an IL-10-like gene were, bovine papular stomatitis virus (BPSV), pseudocowpox virus (PCPV) and parapoxvirus of red deer (PVNZ). BPSV and PCPV are maintained in cattle and like ORFV are zoonoses. PVNZ is a recently classified parapoxvirus (35) and has only been found in red deer in New Zealand. The pathology of the disease caused by BPSV, PCPV and PVNZ resembles ORFV and is confined to the epithelium and oral mucosa (reviewed in (8)). Members of the genus have genomes of 130 to 150 kbp, a G + C content of 64% and show significant cross hybridization.

Initially we tested for cross hybridization of ORFV

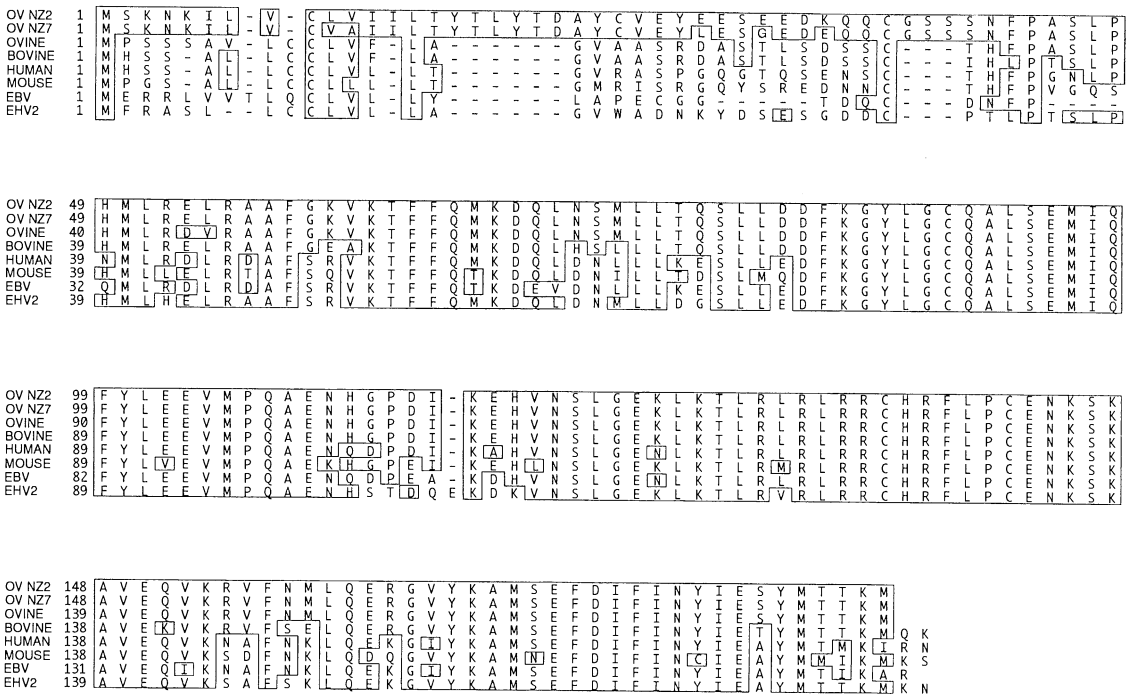

Fig. 4. Alignment of the inferred amino acid sequences of mammalian and viral IL-10. The ORFV NZ2-IL-10 (OV NZ2) and ORFV NZ7-IL-10 (OV NZ7) gene products are aligned with ovine IL-10 (68), bovine IL-10 (69), human IL-10 (39), murine IL-10 (70) EBV IL-10(70), and EHV2 strain T400/3 IL-10 (71). Amino acids identical to those of ORFV NZ2 IL-10 are boxed. (Figure reproduced from ref. 28).

NZ2 IL-10 with BPSV strain V660 (36), PCPV strain VR634 (36) and PVNZ (35). All viruses were grown in primary calf testis cells and viral DNA extracted as described previously (35). Hybridizations were performed over a wide range of hybridization and washing stringencies allowing up to 57% mismatch in nt sequences (37). None of the conditions revealed specific hybridization with BPSV, PCPV or PVNZ sequences. In addition, polymerase chain reactions performed using primers specific for the 3′ and 5′ ends of the coding region of ORFV NZ2 IL-10 and primers based on the highly conserved region of ORFV IL-10 did not reveal IL-10-like sequences. We concluded from these results that it is unlikely that an IL-10-like gene is carried by the parapoxviruses BPSV, PCPV or PVNZ.

Others have shown by restriction endonuclease analysis and DNA/DNA hybridization that ORFV, BPSV, PCPV and PVNZ have distinctive restriction patterns and fail to hybridize over a region of 20 to 30 kb at their termini, despite sharing 80% homology in their central regions (35–36). These observations suggest that although the parapoxviruses are closely related, members of the genus differ significantly within their termini and have apparently evolved different genes within this region which have allowed adaptation to new hosts.

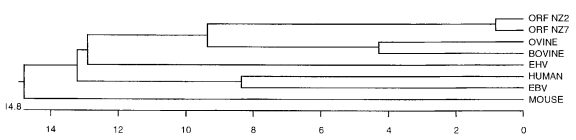

Fig. 5. A phylogenetic map showing the relatedness of ORFV IL-10 with mammalian and herpes virus IL-10 protein sequences.

Functional Activities of Orf Virus IL-10

Mammalian IL-10 is a multifunctional cytokine that has suppressive effects on inflammation, antiviral responses and T-helper type 1 (Th1) effector function (reviewed in ref. 38). IL-10 was initially described as a factor produced by mouse Th2 cells that inhibits the production of cytokines in Th1 cells, suggesting that

IL-10 cross-regulates a type 1 response. This inhibition occurs indirectly through antigen presenting macrophages and dendritic cells but not B cells. IL-10 is produced by various cells including activated monocytes, macrophages, keratinocytes, B cells and $CD8^+$ lymphocytes. It is a potent anti-inflammatory cytokine and a suppressor of macrophage function. In contrast to these immunosuppressive functions, IL-10 is a costimulator of T lymphocytes associated with Th2 responses, mast cells and B cells.

Characterization of the functional activities of EBV IL-10 suggests that viral IL-10-like cytokines have retained only a subset of activities of their cellular counterparts. EBV IL-10 shows cytokine synthesis factor inhibitory activity on mouse and human cells and sustains mouse B cell viability in cell culture, however, it lacks the ability to stimulate mouse thymocyte proliferation, murine mast cell proliferation, or expression of class II MHC antigens on resting splenic mouse B cells (38–39).

We have expressed the ORFV IL-10 gene transiently in COS cells to examine its range of activities in IL-10 *in vitro* assays (28). IL-10 was transfected into COS cells using either the DEAE dextran method (40) or Superfect (Qiagen) and the supernatants containing IL-10 harvested between 48 and 72 h.

Thymocyte Proliferation Assay

Human and ovine IL-10 induce proliferation of mouse thymocytes in the presence of recombinant human IL-2. In light of this activity we examined the effect of ORFV IL-10 in a mouse thymocyte proliferation assay. The results showed that the plasmid containing the ORFV IL-10 like gene expressed a protein with a biological activity, which in this assay, is indistinguishable from ovine IL-10 (Fig. 6) (28). The detection of the activity suggests that the ORFV protein is secreted. Interestingly EBV IL-10 does not induce mouse thymocyte proliferation, which suggests that ORFV IL-10 more closely resembles ovine and human IL-10 than EBV IL-10. Although domains in human IL-10 have been described that are associated with cytokine synthesis inhibition, MHC class II expression and mast cell proliferation (41), a domain which is associated with mouse thymocyte proliferation has not been identified. Our results suggest that such a domain may exist.

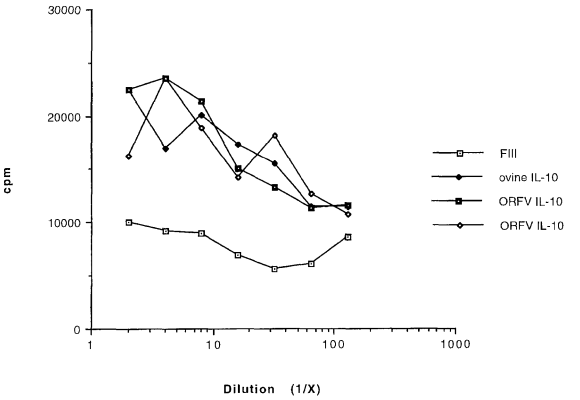

Fig. 6. Murine thymocyte proliferation analysis of ORFV-encoded IL-10. Dilution series of conditioned medium from COS cells, transfected with plasmids shown below, were added to murine thymocytes. Counts per minute represent the incorporation of [^{3}H]thymidine during the final 18 to 24 h of a 120-h incubation. The results shown are the mean of duplicate determinations. FIII, FIII plasmid only (72); ovine IL-10, FIII plasmid containing the ovine IL-10 gene (72); ORFV IL-10, FIII plasmid containing the ORFV IL-10-like gene; ORFV IL-10 (mut), FIII plasmid containing a mutated IL-10-like gene. (Figure reproduced from ref. 28).

Cytokine Synthesis Inhibition Assays

Macrophages and monocytes are major sources of cytokines which can be expressed *in vitro* following stimulation with lipopolysaccharide (LPS) (38). The cytokines produced include IL-1, GM-CSF, tumor necrosis factor (TNF), IL-6, IL-8, IL-10 and IL-12. The production of these cytokines, including IL-10, can be inhibited by IL-10.

We studied the effect of ORFV IL-10 on cytokine synthesis inhibition using ovine macrophages. Alveolar macrophages were obtained from animals by lung lavage. Macrophages were stimulated with LPS at a final concentration of 10 ng for a 24 h period after which time test samples and appropriate controls were added. Supernatants were harvested after 24 h and analyzed for levels of IL-8 and IL-1β using an ELISA and TNF-α using WEHI cells in a bioassay. We have shown that ORFV IL-10 inhibits the production of these cytokines (unpublished data). In addition we have shown that ORFV IL-10 inhibits the production of interferon (IFN)-γ in peripheral blood mononuclear cells (unpublished data).

Functional and Structural Relationships of Mammalian and Viral IL-10 s

An understanding of the functional domains of IL-10 and EBV IL-10 have emerged from studies on the crystal structure of this protein (42–44) and closely related cytokines such as IFN-γ (45–46) and bioassays using synthetic peptides based on IL-10 sequences (41). IL-10 is a dimer composed of identical polypeptide chains and the molecule is predominantly α-helical. Six helices comprising approximately 67% of the structure make up the tightly packed core. Main chain residues 19–36, 56–62, 126–128, and 178 (see human IL-10, Fig. 4) have been described as disordered in the structure. These residues form the flexible regions of the molecule which cluster on opposite ends of the helical bundle and by analogy with IFN-γ are thought to be involved in ligand-receptor interactions. The IFN-γ tertiary structure shows remarkable similarity to IL-10 and peptide mapping (47) and site directed mutagenesis studies of IFN-γ have identified three domains as important for receptor binding (48–49). The basic tail of IFN-γ (N-terminus) has been identified as important for high affinity interactions with its receptors.

IL-10-like activities have been observed with synthetic peptides. These studies have allowed the identification of the functional domains of human IL-10 (41). A nonapeptide with homology to the C-terminal portion of human IL-10 was found to possess activities that mimic those of human IL-10. Some of these activities include inhibition of IL-1β induced IL-8 production by peripheral blood mononuclear cells, down-regulation of TNF-α production by $CD8^+$ T lymphocytes, induction of IL-1 receptor antagonist protein and down-regulation of MHC class II antigen on human monocytes. A nonapeptide representing the near N-terminal region did not reveal cytokine synthesis inhibitory properties, but was found to be a regulator of murine mast cell (MC/9) proliferation. Interestingly only three aa of this peptide are found in the corresponding region of EBV IL-10 and it has been shown that EBV IL-10 does not stimulate the proliferation of MC/9 cells nor does it bind to the IL-10 soluble receptor. The pleiotrophic activities of IL-10 may be related to the multiple functional domains that are present in this molecule or the flexibility that exist within the functional domains. Based on the above studies and the aa sequence of ORFV IL-10, we predict that ORFV IL-10 will have the same immunosuppressive activities as mammalian IL-10s since the domains of IL-10 that are responsible for the immunosuppressive effects are retained in ORFV IL10. In contrast the functional domain that is located at the near N-terminus of mammalian IL-10, which confers mast cell stimulatory activity, is poorly conserved in ORFV IL-10. ORFV IL-10 only shares three aa within this domain, albeit different to EBV IL-10, which suggests that it is unlikely that ORFV IL-10 will have this activity. It has become apparent in the viral IL-10s that domains encoding activities not required for immune evasion are altered.

In Vivo Activities of Orf Virus IL-10

We have constructed a recombinant ORFV in which the IL-10 gene is deleted (unpublished). This has allowed us to characterize the activities of this gene by *in vitro* assay and *in vivo* analysis in a sheep model. A preliminary study of *in situ* cytokine mRNAs in lesions on the skin of sheep infected with either the *wt* ORFV or the IL-10 knock-out recombinant virus has revealed differences in the expression of IFN-γ. The frequency of IFN-γ mRNA-expressing cells is higher in animals infected with the IL-10 knock-out virus compared with animals infected with *wt* virus (unpublished data). A more comprehensive study is underway to confirm this result and extend the range of cytokines analyzed. IFN-γ is mainly produced by NK cells, $CD4^+$ type 1 cells and $CD8^+$ cells. If confirmed, the result suggests that ORFV IL-10 inhibits the production of IFN-γ by NK cells and lymphocytes.

A role for ORFV IL-10 in subversion of specific immunity is suggested by the fact that sheep are susceptible to reinfection with ORFV (6,19). Cell mediated immune responses are thought to be critical in recovery from ORFV infection since humoral antibody appears to play no role (7,50,51). We examined the effect of deleting the IL-10 gene on the protective memory response by infecting animals with either *wt* virus or the IL-10 deleted virus and then 3 months later challenging with ORFV. Both the knock-out virus and the *wt* virus protected animals from the challenge virus and the level of protection was similar in both groups (unpublished data). Further experiments of a longer duration may reveal differences in protective immunity.

The Role of Orf Virus IL-10 in Virulence and Pathogenesis

Our studies on ORFV IL-10 are far from complete but they do provide some basis from which to speculate on the role of this virokine in virulence and pathogenesis. In cases where a virus causes acute infections, the host mechanisms that are most often affected by viral-encoded proteins are the innate and inflammatory responses. The inflammatory response is initiated by the production of pro-inflammatory cytokines IL-1 and TNF at the site of infection. Many poxviruses have been shown to encode factors that block the function of these pro-inflammatory cytokines. In most cases these factors are homologs of cellular receptors for TNF and IL-1. The receptor-like proteins are secreted from virus infected cells and bind cytokines thus acting as decoy molecules.

ORFV replicates in the epidermis where keratinocytes are the principal immune cell. Keratinocytes act as proinflammatory signal transducers responding to non-specific stimuli by secreting inflammatory cytokines, chemotactic factors and adhesion molecules into the extra cellular fluid of the epidermal compartment (52). In the initial phase of non-specific cutaneous inflammation, keratinocytes release IL-1 and TNF-α. IL-1 and TNF-α activate dermal vascular endothelium, which upregulates the expression of adhesion molecules involved in the recruitment of leukocytes to the endothelium. In conjunction with chemokines, such as IL-8, these cytokines direct the migration of leucocytes from the circulatory system into the epidermis. Recent evidence shows that TNF-α and perhaps other pro-inflammatory cytokines are down-regulated in keratinocytes by IL-10 (53). An inverted relationship between IL-10 and TNF-α levels was observed in supernatants of CD-23 stimulated human keratinocytes and neutralisation of IL-10 with anti-IL-10 mAb increased in both magnitude and duration TNF-α production by keratinocytes through CD23 ligation. These observations strongly suggest that keratinocytes may be the main target of ORFV-encoded IL-10 during the early stages of cutaneous inflammation and that ORFV IL-10 inhibits the production of pro-inflammatory cytokines in these cells. Furthermore, the production of pro-inflammatory cytokines secreted by immigrating macrophages and $CD8^+$ cells are likely to be blocked by ORFV IL-10.

IFN-γ plays an important regulatory role in the inflammatory response and poxviruses block this cytokine by producing soluble IFN-γ receptor-like proteins. We have no evidence that ORFV encodes a receptor for IFN-γ but ORFV may have the potential to suppress the production of IFN-γ since this cytokine is inhibited indirectly in NK and $CD4^+$ Th1 cells by IL-10. It is not known whether the inhibition of IFN-γ in $CD8^+$ cells is direct or indirect. In addition, we have found a further factor encoded by ORFV that could potentially act in concert with IL-10 to reduce inflammation namely a homolog of a chemokine binding factor (unpublished).

In addition to their roles in inflammation, TNF-α and IFN γ are involved in the anti-viral innate responses and specific early immune responses. TNF-α inhibits viral replication and induces apoptosis in virus-infected cells and IFN-γ acts synergistically to enhance the antiviral cytotoxic activity of TNF-α and the anti-viral activities of IFN-α and IFN-β. We have found no evidence that ORFV encodes homologs of the IFN-γ, IL-1 or TNF-α receptor-like proteins that are common in other poxviruses, and the discovery of an IL-10-like cytokine and a number of other genes in the termini of ORFV that do not have homologs with sequences in the data base (unpublished data), suggests that ORFV has evolved alternative strategies to suppress inflammation and the innate responses.

The endogenous expression of IL-10 has been measured during immune responses induced by a variety of infectious agents (reviewed in ref. 38). In some instances high levels of IL-10 expression and associated Th2-like responses have been observed in circumstances in which these responses may be inappropriate. Much of this work has been done *in vitro*, where cells have been derived directly from animals and humans and stimulated in short-term culture. Examples of this correlation include mice infected with the retrovirus causing murine acquired immunodeficiency syndrome (54) and the response against HIV when the patients immune system begins to collapse (55). This correlation has been shown more directly by analyzing the production of cytokine mRNAs in tissue. Examples include (a) the analysis of mRNAs derived from leprosy lesions, a lepromatous form of which is associated with high levels of antibody production and a tuberculoid form of which is associated with DTH reactions (56) and (b) analysis of mRNAs in tissue derived from susceptible and resistant strains of mice infected with *Leishmania*.

(57–58). These studies showed that the expression of IL-10 correlated with the expression of other Th2 cytokines which in turn correlated with susceptibility to infectious agents that are more effectively eliminated by a cell mediated response.

Recent studies demonstrate that sheep produce a potent cellular response to ORFV infection (7,59–60). However, the response could be viewed as unusual for a virus infection as $CD4^+$ T cells were found at higher levels than $CD8^+$ T cells or B cells in afferent and efferent lymph draining from the site of infection. It was expected that the $CD8^+$ T cell response might have been numerically more significant if $CD8^+$ cytotoxic cells were important in containing the virus, and the observation might indicate that ORFV has acquired a mechanism for preventing the accumulation and activation of $CD8^+$ T cells. These findings are consistent with the effect of IL-10 on cellular immunity and analogous with the immune response observed in humans infected with EBV. Primary EBV infections in adults is associated with substantial dysfunction in both T and B cell compartments of the immune system (61). Moreover herpes viruses are well known for their ability to establish latent and persistent infections and it is thought that viral-encoded proteins that counteract the host immune defences, in particular viral IL-10 which inhibits IFN-γ synthesis in T cells, is likely to play a role in the establishment of such infections (62). These observations suggest that there may be a link between persistent ORFV infections seen in some animals and the expression of a viral IL-10.

An unusual feature of the ORFV lesion is the accumulation of MHC Class II^+ dendritic cells (10,18). Phenotypically > 90% of these resemble a subpopulation of dermal dendritic cells found in normal skin which are MHC ClassII^+, CD1-, CD11b- and CD11c-. These cells are not phenotypically related to either MHC Class II^+ epidermal Langerhans cells which express the CD1 antigen or tissue macrophages however a role in antigen presentation cannot be excluded. IL-10 and vascular endothelial growth factor have been shown to interfere with dendritic cell maturation and function (63–64) while GM-CSF and TNF-α are associated with dendritic cell recruitment, survival, growth, differentiation and activation (65–66, reviewed in ref. 67). The discovery of IL-10 and VEGF-like genes in ORFV and a gene that apparently inhibits GM-CSF raises the possibility that ORFV encoded factors have a role in the accumulation and apparent dysfunction of a largely uncharacterized population of dendritic cells.

The striking homology of ORFV IL-10 to ovine IL-10 strongly suggests that the viral gene represents a processed ovine gene captured by the virus at a late stage in its evolutionary development. Further studies are under way to further characterize the activities of ORFV IL-10 and to determine its role in pathogenesis and virulence.

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Myxoma Virus Expresses a TNF Receptor Homolog with two Distinct Functions

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Abstract. Myxoma virus, a member of the poxvirus family of DNA viruses, encodes many virulence factors to combat and evade the host immune responses. Among the virus-encoded immuno-modulators is M-T2, a tumor necrosis factor receptor (TNF-R) homologue. M-T2 is secreted as monomeric and dimeric species that bind and inhibit rabbit TNF in a species-specific manner. Deletion analysis indicates that the anti-TNF function is mediated by the first three of four cysteine rich domains (CRDs) of M-T2. In addition, the intracellular form of M-T2 has the ability to block virus-induced apoptosis in lymphocytes, and the first two CRDs appear to be sufficient for this function. Although the mechanisms for the anti-TNF and anti-apoptotic functions of M-T2 are not yet fully defined, we postulate that these dual activities of M-T2 are mediated through different functional motifs and abrogate distinct cellular responses to virus infection.

Key words: myxoma virus, immuno-modulator, viroceptor, TNF receptor, apoptosis

The Virus as Rosetta Stone

Viruses are often regarded simply as infectious agents responsible for a variety of destructive diseases. An alternate view is to look upon viruses as biological probes for the study of the sophisticated immune network (1–4). In this view, viruses are keys to unlocking the complex secrets of the mammalian immune system. In defending against viral assault, vertebrate hosts deploy aspects of their complex immune system in a multi-pronged attack aimed at eliminating a wide variety of invading viruses. Against this powerful array of immune modalities, viruses have successfully co-evolved a variety of strategies to circumvent the host immune responses. For example, viruses with smaller genomes usually ensure their survival by exploiting the weakness or gaps in the host immune repertoire. Larger DNA viruses, particularly poxviruses, herpesviruses and adenoviruses, have taken the approach of encoding a range of viral proteins aimed at systematically disabling or dismantling aspects of the host immune response (5–7). These viral gene products, termed immuno-modulators, can be grouped into several categories based on their targets and mechanisms of action. *Virokines* refer to secreted viral proteins that mimic host cytokines in order to promote growth, act as competitive inhibitors or to favor an immune response that is beneficial to the virus. *Viroceptors* are secreted or cell surface viral proteins homologous to cellular receptors, that in some cases can competitively bind cytokine ligands and thereby block immune and inflammatory signals. They usually function as antagonists to preclude host ligands from interacting with the true cellular receptors and thus disarm the subsequent immune responses, but examples of viroceptors capable of active signaling are also known. A number of viruses employ two additional mechanisms to evade detection and promote virus survival. The first of these is the downregulation of cell surface immune markers, such as the class 1 major histocompatibility complex (MHC I) or CD4, thereby preventing the immune system from detecting the presence of the infection.

Termed *virostealth*, this strategy seeks to prevent the infected cell from communicating its aberrant state to the cellular immune system. The last mechanism widely employed by viruses, *viromitigation*, is exemplified by the blocking of apoptosis or programmed cell death which many cells will undergo upon virus infection (8,9). These four categories of viral immuno-modulators combine to systematically evade or compromise those aspects of the host immune and inflammatory responses that are detrimental to virus replication. The large DNA viruses have extensive genetic encoding capacity which enable them to adopt more than one of these strategies. Interestingly, a single anti-immune strategy can often be employed by a variety of viruses using related or unrelated proteins.

Among the many viral immuno-modulators, some were derived from cellular homologs that were hijacked by viruses during their co-evolution with the host, whereas others are only detected in viruses. It is possible that the corresponding cellular components for these latter molecules have not been identified to date, but will be uncovered as the human genome sequencing project unfolds. The study on viruses, therefore, not only enhances our knowledge of pathogenesis in order to better control viral diseases, but also facilitates a greater understanding of immunology and other related fields. Thus, the study of viral immuno-modulators has been compared to the use of the Rosetta stone to decode the complexities of Egyptian hieroglyphics (10).

Poxviruses have large linear double-stranded (ds) DNA genomes that range within 130–300 kilobase pairs (kbp) in length and have hairpin termini. The virion is a brick-shaped oval 200 to 400 nm long (11,12). Unlike other eukaryotic DNA viruses, poxviruses replicate exclusively in the cytoplasm of the infected cells and encode their own transcription factors and replication machinery. The genes for virus replication and virion assembly are clustered within the central region of the genome. Open reading frames (ORFs) found towards the terminal inverted repeats (TIRs) of the genome are often dispensable for virus growth in tissue culture, but contribute to determining tissue specificity, host range and virulence (13). Since these viral proteins play important roles in subverting host immune network, they have been extensively studied in the last few years (5–7). In this review, we focused on M-T2, a tumor necrosis factor (TNF) receptor homologue, as the prototypic example of a viral immuno-modulator encoded by one particular poxvirus (myxoma virus).

Myxoma Virus and its Encoded Immuno-Modulators

Myxoma virus was originally identified as the causative agent of myxomatosis, a lethal disease of European rabbits (*Oryctolagus cuniculus*) first described at the end of the 19th century (14). Interestingly, myxoma virus is not fatal to its natural hosts, the North American brush rabbit (*Silvilagus bachmani*) and the South American tropical forest rabbit (*Silvilagus brasiliensis*) (14,15), suggesting that the genetic diversity amongst different rabbit hosts affects the virulence of the virus. In the early 1950s, myxoma virus was deliberately introduced into Australia to control the rampant populations of feral European rabbits. Initially, this achieved the desired result and a large number of rabbits died in the first year following virus release. However, the surviving rabbits assumed greater resistance to myxomatosis while the myxoma virus itself simultaneously became more attenuated, resulting in a rapid resurgence of the rabbit populations (14,15). Myxoma virus therefore provides a very informative model to study the co-evolution and interaction between viral anti-immune molecules and host anti-viral systems (16,17).

The 165 kbp myxoma DNA genome has now been fully sequenced (18), and several important virus virulence genes have been mapped and characterized to date (Fig. 1). Some of the virulence factors studied to date are found to exist as two copies in the 10 kbp terminal inverted repeats TIRs. The virulence factors studied, and their respective strategies against the host immune network are detailed in Table 1 (13,17). SERP-1 is a secreted serine proteinase inhibitor which plays an anti-inflammatory role in virus infection (19,20), and the purified protein retains this property in several animal models of inflammation (21,22). Secreted myxoma growth factor (MGF) is a *virokine* that mimics cellular epidermal growth factor (EGF) to stimulate target cells into mitogenesis (23,24). M-T2 and M-T7 share sequence homology with the cellular receptors for TNF and interferon-gamma (IFNγ), respectively. By binding to the host ligands, these soluble *viroceptors* disrupt the anti-viral activities of those cytokines (25,26). In order to maximally utilize its coding

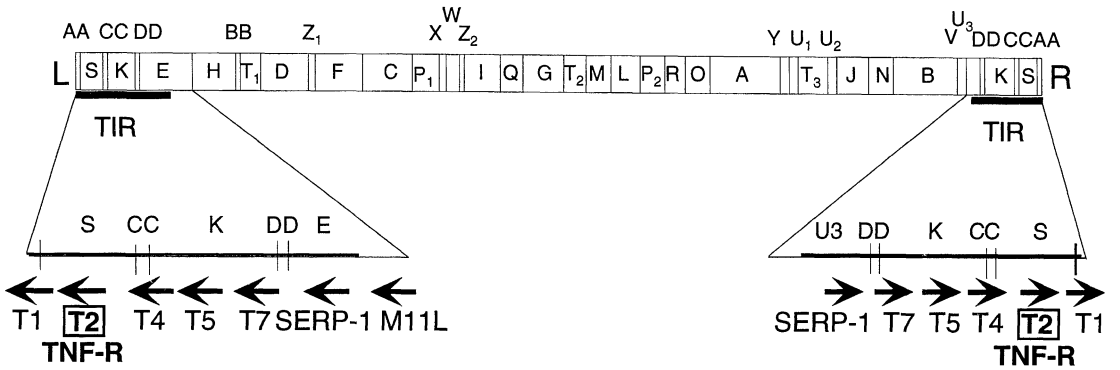

Fig. 1. Genomic location of myxoma genes implicated as virulence factors. The BamH1 map of the myxoma virus 165 kbp genome (fragments A to DD) is displayed. The terminal inverted repeats (TIRs) comprise 10–11 kbp at each end of the genome, and contain most of the virulence factors reported to date, including the TNF-receptor homolog, T2.

capacity, myxoma virus also expresses viral proteins with multiple functions. For instance, in addition to the anti-IFNγ activity, M-T7 can act as a chemokine-binding protein (27). Chemokine binding function has also been attributed to M-T1, however the mechanisms by which M-T1 and M-T7 act appear to be different (27,28). M-T2 is another example of a single viral protein with a dual function in that it both inhibits extracellular TNF, and acts intracellularly as a *viromitigator* to prevent virus infected T-cells from undergoing apoptosis (29). Other myxoma virus viromitigators that block apoptosis include M-T4 (an endoplasmic reticulum-retained glycoprotein), M-T5 (an ankyrin-repeat containing host range protein) and M11L (a bcl-2 like protein) which all act in conjunction with M-T2 to permit replication in infected lymphocytes (29–31).

Finally, infection of myxoma virus causes a dramatic decrease of cell surface class 1 MHC molecules (32) and CD4 from infected $CD4^+$-T cells (33). In contrast, only moderate levels of down-regulation of MHC-I was observed with either Shope fibroma virus (SFV, a related but benign leporipoxvirus) or vaccinia virus (an attenuated

Table 1. Myxoma virus proteins implicated as virulence factors

Myxoma Gene	Copy Number	Level of Action	Other Poxvirus Homologs	Cellular Homologs	Anti-immune Strategies
SERP-1	2	*virokine*	CPV Spi-3	Serpin superfamily	Inhibits inflammatory response
MGF	1	*virokine*	Other poxvirus growth factors	EGF/TGFα	Mimics cellular growth factors and stimulates cells into mitogenesis
M-T2	2	*viroceptor & viromitigator*	SFV S-T2 CPV crmB/C/D	TNF receptor	Binds and inhibits cellular TNF Inhibits T-lymphocyte apoptosis
M-T7	2	*viroceptor*	T7 family[a]	INF-γ receptor	Binds cellular INF-γ and chemokines
M-T1	2	*viroceptor*	35 kDa family[b]	None known	Binds β-chemokines (CC-class) and inhibits leukocyte infiltration
M-T5	2	*viromitigator*	T5 family[c]	None known	Block apoptosis of virus-infected lymphocytes
M-T4	2	—	T4 family[d]	—	—
M11L	1	—	SFV-S11L	—	—

[a]SFV S-T7, VV B8R, Variola virus B9R, Swinepox virus C6L.
[b]RPV 35 kDa, VV C23L/B29R, Variola virus G3R, CPV D1L/H5R, SFV S-T1.
[c]SFV S-T5, VV B4R, CPV B3R.
[d]SFV S-T4, Capripox virus T4, VV-WR B9R.

orthopoxvirus). It is expected that more virus immuno-modulators will be discovered as more myxoma virus genes are sequenced and characterized.

TNF and Cellular TNF Receptor Superfamilies

Tumor necrosis factor was first discovered on the basis of its tumoricidal activity (reviewed in (34–37)). Distinct, but related ligands, originally referred to as TNFα and TNFβ (now known as TNF and lymphotoxin α/β or LTα/β) were subsequently identified (34,35). Since then the TNF ligands have been intensively studied and shown to play pivotal roles in many immune responses (36,37). For example, TNF can directly mediate cytotoxicity, affect cell growth and differentiation, regulate T cell activation/proliferation and B-cell co-stimulation, modulate the cell surface expression of MHC and adhesion molecules, and induce the expression of many other pro-inflammatory cytokines. From the point of view of the virus, an important feature of TNF is the potent anti-viral activity of this ligand family (38).

In recent years, the TNF family has expanded to include at least 10 pleiotropic cytokines, such as NGF, TRAIL (TNF-related apoptosis-inducing ligand) and ligands for Fas, CD40, CD30, CD27, 4-1BB and OX40 (35–37). Although most are type II transmembrane proteins, soluble trimeric forms of TNF, FasL and CD40L released by proteolytic cleavage also exist, though these soluble forms in some cases have lower activity than their membrane-bound counterparts (37,39). Sequence comparison of TNF family members reveals that the C-terminal regions (about 150 aa long) are well conserved. This extracellular region is involved in binding to the cognate receptors, while the intracellular N terminal domains are poorly conserved between different TNF members (35,37).

The receptors that engage the TNF ligands form the still-growing TNF receptor (TNF-R) superfamily which includes cellular TNF-R1 (p55), TNF-R2 (p75), LT-R, Fas, NGF-R (p75), CD40, CD30, CD27, 4-1BB, OX40, human HVEM, chicken CAR1, Wsl-1/DR-3 (39) and many recently identified molecules, such as the death receptor group of TRAIL, DR4/TRAIL-R1 (40), DR5/TRAIL-R2 (41) and TRID/DcR1/TRAIL-R3 (41–43) (Fig. 2). TNF receptor homologs are even found in plants. For example, a TNF-R family protein, called CRINKLY4 was recently identified in maize (corn) and found to have a kinase activity involved in plant epidermal cell differentiation (44). The cellular TNF-Rs are extensively reviewed elsewhere (35,37,39); here we will focus only on the virus counterparts.

Although soluble forms of some TNF-Rs exist with varying ligand affinities compared to the membrane-associated ones, most cellular TNF-Rs are type I membrane proteins that have a conserved N-terminal region, a single transmembrane segment and short cytoplasmic C-terminal region (35,39). The extracellular ligand binding domains contain from two to six conserved cysteine rich domains (CRDs). Each CRD contains approximately 40 amino acids, including 6 conserved cysteine residues. Deletion analysis clearly indicates that the CRDs are essential for the ligand-receptor interaction. The cytoplasmic domain ranges from 46 to 221 residues in length and varies greatly between TNF-R family members. No enzymatic activity has been associated with any of the TNF-Rs, except for the plant CRINKLY4 protein. Instead, the receptors appear to act by association with intracellular factors that trigger diverse signal transduction cascades, consistent with the finding that TNF superfamily members establish diverse inflammatory and immune responses through their respective receptors (35). The only known common signal transduction action of the TNF-R members is that several (TNF-R1, Fas, DR3, DR4 and DR5) share a death domain that is involved in signal transduction of programmed cell death (39–41).

The crystal structure of human TNF in conjunction with the receptor (p55) complex (45–47) confirm that soluble TNF and LTα/β function as trimers to bind and then functionally cluster the receptor molecules, thereby activating the subsequent signaling cascade (Fig. 5a) (35). Since all TNF-R superfamily members share high homology in the conserved CRD region, a similar receptor oligomerization is postulated to occur with other members of the superfamily upon the binding of their appropriate ligands.

Viral TNF Receptor Homologs

TNF orchestrates powerful anti-viral responses by a variety of mechanisms, including the direct killing of infected cells (cytolysis), induction of apoptosis and the inhibition of viral replication (38,48). It is not surprising, therefore, that many viruses have acquired

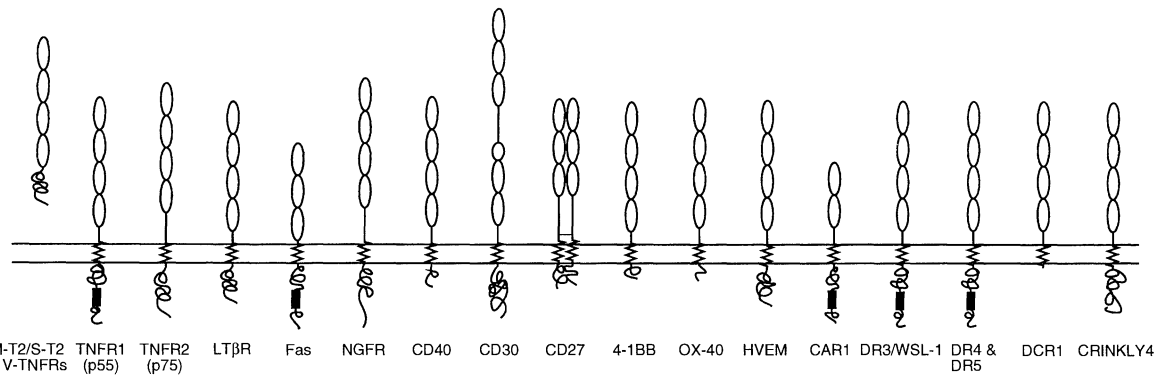

Fig. 2. Schematic representation of the TNF receptor superfamily (modified from (35)). The number of CRDs (ovals) is indicated for each receptor. The presence of a pro-apoptotic intracellular death domain is shown (black box) for TNFR1, Fas, CAR1 and DR3, 4, 5. Note that none of the viral TNF receptors (v-TNFRs) possess transmembrane domains characteristic of the cellular receptors.

specific mechanisms to counteract this cytokine. For example, adenoviruses encode four sets of proteins to subvert the anti-viral activities of TNF (49). Among the poxviruses, members of the orthopoxvirus family encode a serpin designated crmA or SPI-2 which inhibits both TNF- and Fas-induced apoptosis by blocking intracellular caspases (50).

In 1991, the first virus encoded TNF-R homologue, S-T2, was discovered in Shope fibroma virus (SFV) (51,52). The S-T2 gene had first been identified and named for its location as the second open reading frame in the TIR of the SFV genome (53,54), but at that time a homology search of the existing databases only identified the related low affinity nerve growth factor receptor (NGF-R) (55). The relationship of S-T2 to the TNF-R superfamily was not revealed until the type I and type II human TNF-Rs were cloned and sequenced (51). Shortly thereafter, the importance of S-T2 was confirmed by the fact that recombinant S-T2 expressed and secreted from Cos cells exhibited the capacity to bind and inhibit TNF (52).

Related T2-like genes that encode viral TNF-R homologs have subsequently been characterized in many members of the poxvirus family including myxoma virus M-T2 (25), cowpox virus (CPV) crmB (56), crmC (57) and crmD (58), variola virus G2R (59,60), and vaccinia virus A53R, C22L/B28R (61) and SalF19R (62) (Table 2). Most vaccinia virus strains examined so far only encode disrupted and nonfunctional TNF-R-like ORFs. For example, a frameshifted ORF (A53R) and two copies of prematurely truncated ORFs (C22L and B28R) in vaccinia virus strain Copenhagen share some homology with the CRDs of cellular TNF-Rs (61).

Table 2. Poxvirus TNF-Receptor homologs

Gene/ORF	Virus (Strain)	Characteristics	Protein Function	References
M-T2	Myxoma virus	Secreted (Early gene)	Binds and inhibits rabbit TNF	(25,65)
S-T2	Shope fibroma virus	Secreted (Early gene)	Binds and inhibits TNF	(52)
CrmB	Cowpox virus	Secreted (Early gene)	Binds and inhibits TNF and LT-α	(56)
CrmC	Cowpox virus	Secreted (Late gene)	Binds and inhibits murine TNF and LT-α	(57)
CrmD	Cowpox virus (Brighton Red)	Secreted (Early gene)	Binds and inhibits TNF and LT-α	(58)
D2L/H3R	Cowpox virus (GRI-90)	351 aa	Not tested	(64)
D13L		111 aa		
A53R		186 aa		
K2R		322 aa		
K3R		167 aa		
A53R	Vaccinia Virus (Copenhagen)	Fragmented	No known activity	(61)
C22L/B28R		Prematurely truncated		
Sal F19R	Vaccinia Virus (WR)	Prematurely truncated	No known activity	(62)
G2R	Variola major virus (Bangladesh 1975)	348 aa	Binds and inhibits TNF and LT-α	(59,60)

Similar discontinuous ORFs (SalF19R) were also found in vaccinia strain WR (62). In contrast, the related but highly virulent causative agent of smallpox, variola virus, encodes two copies of functional TNF-R homologs (59,60,63). It is speculated that the loss of functional TNF-R copies in vaccinia virus, along with its attenuated virulence, are due to the lack of *in vivo* selective pressure since vaccinia has been passaged out of true vertebrate hosts for over two centuries (11).

CPV (Brighton Red strain) has been found to encode three distinct TNF-R homologs, referred to as crmB, crmC and crmD. Amongst them, crmB is an early viral gene product secreted from the infected cells, shares 48% identity with both S-T2 and M-T2, and can bind and inhibit TNF and LTα (56). CrmC is expressed at late times of infection as a soluble secreted TNF-R homologue that lacks the C-terminal domain conserved within the other T2-like molecules. CrmC specifically binds and inactivates mouse TNF, but not mouse LTα or human TNF (57). In addition, a new member termed crmD was recently characterized in CPV (Brighton Red strain) sharing 50% and 43% identity with crmB and S-T2 respectively. *In vitro* analysis has shown that this early viral protein is capable of binding and blocking TNF and LTα. Interestingly, crmD is absent from most CPV strains and many other orthopoxviruses, but is found in four strains of ectromelia virus which do not encode crmB or crmC (58). Another CPV strain (GRI-90) has five distinct ORFs that show variable homology to TNF-R (64). Myxoma virus encodes two copies of a TNF-R homologue, termed M-T2, each of which maps as the second ORF of the TIRs (25). Sequence analysis shows that M-T2 shares 75% amino acid identity with S-T2 and 47% and 41% identity with crmB and crmD, respectively. The biochemical characterization and functional activities of M-T2 are discussed in the next section.

All the viral TNF-Rs exhibit sequence homology specifically with the N-terminal ligand binding CRD region of the cellular receptors (55). However, unlike their cellular counterparts, most poxviral TNF-Rs identified so far do not have a transmembrane domain, but instead, by virtue of signal sequences at the N-terminus, most are believed to be secreted from infected cells. The C-terminal 140 amino acids of the viral TNF-Rs bears no similarity to cellular TNF-Rs or any other known proteins in the database. Nevertheless, this region is highly conserved between S-T2, M-T2, crmB and crmD (Fig. 3), suggesting that this domain may have a conserved function and perhaps has evolved from another, as yet unidentified, cellular counterpart (55).

M-T2 is a Virus Encoded Antagonist of Rabbit TNF

The activity of M-T2 against TNF has been examined by measuring the ability to block TNF-induced cytotoxicity of the TNF-hypersensitive L929-8 cell line. M-T2 was found to block L929 cytolysis induced by rabbit TNF, but not human or murine TNF, suggesting that M-T2 is species specific (65). Scatchard analysis demonstrated that M-T2 binds to rabbit TNF with high affinity (K_d = 170–195 pM) (66), comparable to that of cellular TNF receptors (67,68). Consistent with the cytolysis data, M-T2 has a much lower binding affinity (K_d = 1.7 nM) for murine TNF and no measurable interaction with human TNF (66).

Biochemical characterization revealed that expression of M-T2 is driven by an early promoter (25,66). Despite the predicted 35 kDa size, secreted M-T2 migrates as a 55–60 kDa protein on denaturing SDS-PAGE (25). This discrepancy is due to glycosylation which is predicted to occur at several of the four putative N-linked glycosylation sites and one putative O-linked glycosylation site (69). M-T2 is secreted from the virus infected cells as a monomer and a dimer, and sedimentation equilibrium analysis indicates that the observed mass of the purified monomeric form is about 40 kDa while the disulphide-linked dimeric form is around 80 kDa (66). Although both the monomeric 40 kDa and dimeric 80 kDa forms are capable of binding rabbit TNF with comparable affinities, the dimer is a more potent inhibitor of TNF-induced cytolysis (66). The fact that most TNF-Rs oligomerize following binding of their cognate ligand trimer may explain why the homodimeric form of M-T2 is more effective than the monomer at preventing TNF signaling through the TNF-R.

Deletion Analysis of M-T2

To map the domains in M-T2 that are responsible for TNF binding activity, a series of C-terminal truncation

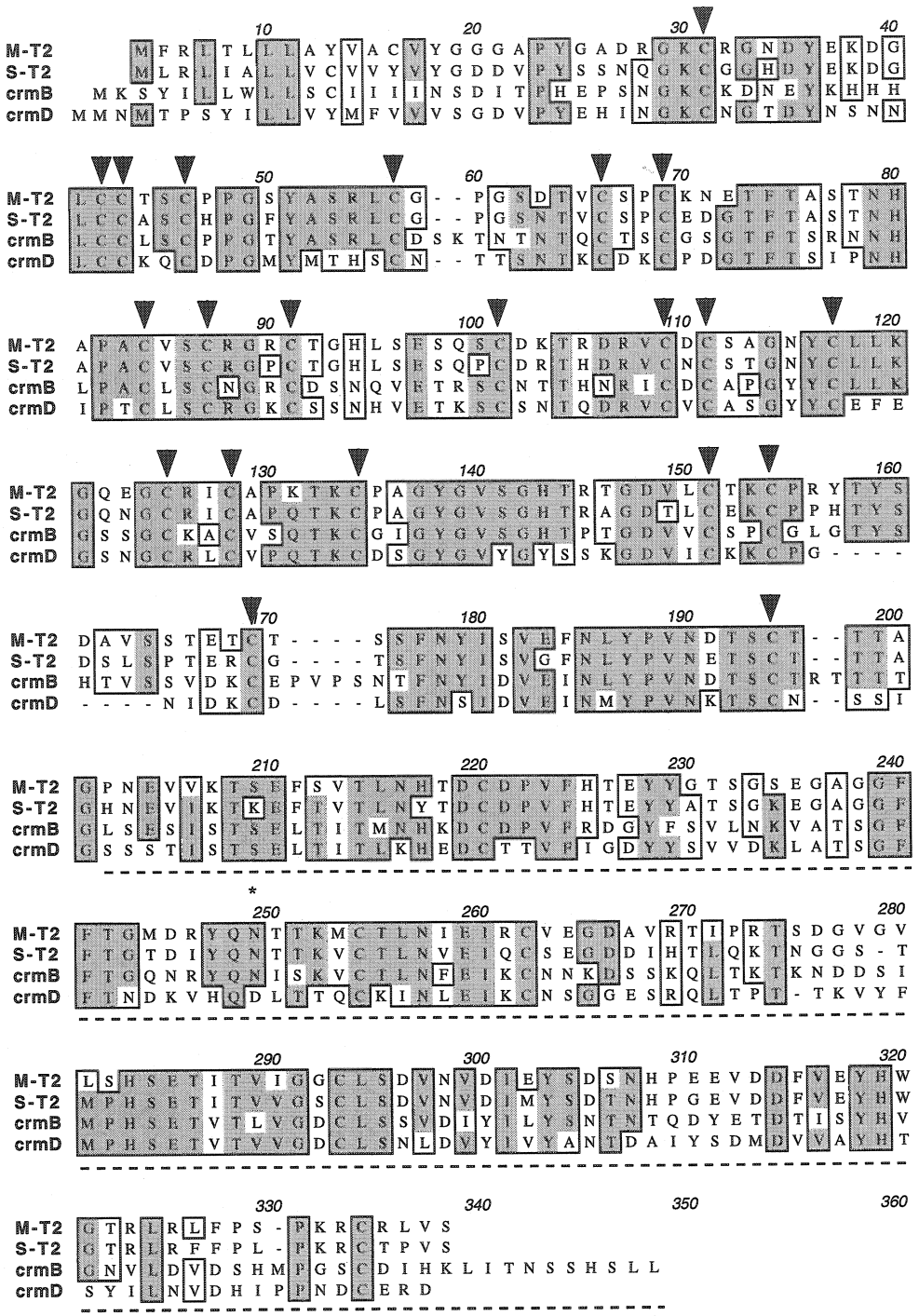

Fig. 3. Sequence comparison of poxvirus TNF receptor homologs from myxoma virus (M-T2), Shope fibroma virus (S-T2), and cowpox virus (crmB and crmD). Areas of sequence identity are boxed and shaded in grey while conserved regions are in open boxes. Conserved cysteines within the CRDs are indicated with arrows and the conserved C-terminal tail region is underscored with a dashed line. Genebank accession numbers are: M95181/M37976 (M-T2); A23727 (S-T2); U90225 (crmB); and U87234 (crmD).

and CRD deletion mutants of M-T2 were constructed and expressed from recombinant vaccinia viruses (Fig. 4). Unexpectedly, all of the M-T2 mutants were poorly secreted from the infected cells in comparison with wild type M-T2, despite the presence of identical signal sequences (69), suggesting that the C-terminal domain of M-T2 is required for proper secretion and trafficking. Removal of only the last 24 amino acids of the C-terminus (MT2-ΔD303) results in effective intracellular retention of the variant protein. It is still unclear why the C-terminal sequence of M-T2 has such a profound effect on efficient protein secretion. However, as none of the cellular receptors contains this C-terminal domain, despite close sequence conservation among the related poxvirus members (such as S-T2 and crmB), this may indicate the presence of other unidentified function(s) of T2-family of proteins (55).

Co-immunoprecipitation studies and cytolysis assays were used to investigate the interaction between M-T2 mutants and rabbit TNF. Both studies demonstrated that the three N-terminal CRDs are required for M-T2 to bind and inhibit TNF. The deletion of the 3rd cysteine-rich domain (MT2-ΔL113) eliminates the ability to bind and inhibit TNF, whereas MT2-ΔN169 (which excises only the fourth CRD, but keeps the first three intact), retains TNF binding and inhibition (Fig. 4). In addition, excision of any one of the first three CRDs abrogates TNF binding (Fig. 4) (69). Thus, the N-terminal three CRDs are essential for M-T2 to bind rabbit TNF and competitively inhibit its interaction with cell surface

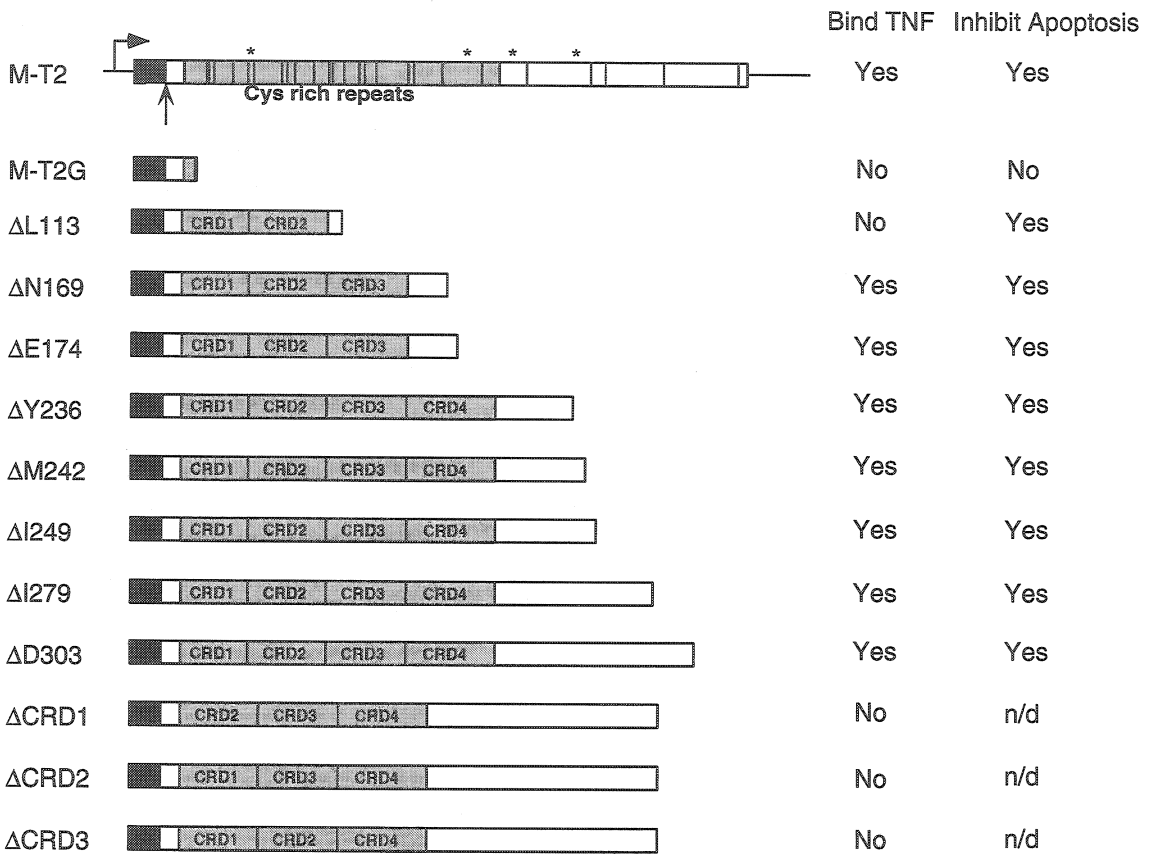

Fig. 4. Schematic representation of M-T2 and M-T2 truncation and deletion mutants. The positions of the conserved cysteines (vertical black lines) within the CRDs (grey boxes) of M-T2 are shown. The signal sequence (black box) is cleaved as indicated (arrow), and the mature protein is secreted and glycosylated (putative N-glycosylation sites indicated by *). M-T2 truncations are labeled according to the C-terminal residue of the truncated protein (single letter code and position), or according to which CRD has been deleted. n/d indicates that the experiment was not completed.

TNF-Rs, thereby preventing subsequent signal transduction.

M-T2 is an Important Virulence Factor of Myxoma Virus

Since TNF is a critical anti-viral cytokine, it was predicted that M-T2 might contribute to the virus virulence, by binding and inhibiting rabbit TNF. This was tested by the targeted disruption of both copies of the M-T2 gene in myxoma virus. The resulting M-T2 minus recombinant virus, termed vMyxT2G, replicates normally in permissive fibroblast cell lines, confirming that M-T2 does not affect the virus growth in tissue culture (25). The wild type parental virus (vMyxlac) and the recombinant M-T2 minus (vMyxT2G) myxoma viruses were tested for pathogenesis in immunocompetent European rabbits. vMyxlac caused typical clinical symptoms of myxomatosis with 100% lethality in the infected rabbits within 10–11 days, while vMyxT2G induced a considerably attenuated disease phenotype with primary and secondary lesion sizes smaller than those in the control rabbits infected with vMyxlac. Supervening Gram-negative bacterial infections also occurred to a lesser extent, and among the vMyxT2G infected rabbits less than 40% succumbed to the disease, while the remaining rabbits completely recovered within 30 days and showed resistance to further challenge with wild type myxoma virus (25). Thus, M-T2 is an important virulence factor in contributing to the disease outcome of myxomatosis.

M-T2 is also an Intracellular Apoptosis Inhibitor

An important aspect of myxomatosis is that myxoma virus is able to productively replicate in lymphocytes and disseminate to secondary sites via the lymphatic channels, to establish a systemic infection (17,70). Virus growth analysis demonstrated that both wild type (vMyxlac) and T2 minus (vMyxT2G) myxoma viruses replicate equally well in a rabbit fibroblast cells (25). However, while wild-type myxoma virus is able to productively infect RL5 $CD4^+$ T lymphocytes, vMyxT2G cannot, due to the fact that vMyxT2G infected RL5 cells undergo rapid programmed cell death or apoptosis (29). It was found that vMyxT2G infected lymphocytes undergo apoptosis as early as 3 h post-infection (p.i.), and over 40% of vMyxT2G infected cells were observed to be undergoing late-stage apoptosis by 6 h p.i. (29). vMyxT2G infection was also found to specifically cause apoptosis of non-adherent primary peripheral blood leukocytes but not adherent monocytes (L. Sedger and G. McFadden, unpublished data). These findings indicate that M-T2 has an additional role in determining host range by preventing apoptosis of infected lymphocytes.

Besides M-T2, three other myxoma virus proteins, M11L (29), M-T4 (31) and M-T5 (30), have been shown to exhibit anti-apoptotic properties (8). Targeted deletion of any one of these viral genes results in a dramatic attenuation of the virus and the loss of the ability to productively replicate in lymphocytes. Typical apoptotic features such as cell shrinkage, membrane blebbing, nuclear DNA condensation and fragmentation are also observed in T-lymphocytes infected by each of these mutant viruses (29–31). Interestingly, each of these anti-apoptotic molecules is localized in different cellular compartments: M-T2 is detected as both extracellular and intracellular forms (71); M-T4 is strictly retained in the endoplasmic reticulum (31); M-T5 is cytosolic (30); and M-11L is associated with still-undefined intracellular membranes (H. Everett and G. McFadden, unpublished data). Although the precise mechanisms utilized by these anti-apoptotic proteins remain to be defined, it is speculated that they may target different signaling pathways since the absence of any one of these viral genes results in apoptosis of infected RL5 cells.

Since M-T2 can bind to and inhibit rabbit TNF, it was initially speculated that extracellular M-T2 might block TNF mediated apoptosis of myxoma-virus infected T-lymphocytes. However, further studies revealed that the anti-apoptotic activities of M-T2 are intracellular and do not involve TNF inhibition. For example, no trace of rabbit TNF was detected in the RL5 culture medium (M. Schreiber and G. McFadden, unpublished data), and neither infected nor uninfected RL5 cells are sensitive to cytolysis or apoptosis mediated by rabbit TNF (M. Barry and G. McFadden, unpublished data). Furthermore, addition of exogenous purified M-T2 protein (5 ug/ml) into cell culture could not rescue the vMyxT2G infected RL5 cells from undergoing apoptosis even though the same concentration of M-T2 is potent at blocking rabbit TNF induced cytolysis of L929-8 cells (29). This

further suggests that M-T2 acts intracellularly, rather than extracellularly, to block apoptosis of infected lymphocytes.

In order to map the anti-apoptotic domain in M-T2, a series of C-terminal truncated M-T2 recombinant myxoma viruses were constructed and tested for their role in protecting infected lymphocytes from undergoing apoptosis (Fig. 4) (71). Surprisingly, all these mutant viruses, including MT2-ΔL113, which had previously been shown to be incapable of binding and inhibiting TNF, had the ability to block apoptosis in virus-infected RL5 cells (Fig. 4) (69,71). It appears, therefore, that TNF-inhibition and anti-apoptotic functions are mediated by distinct mechanisms. In contrast to TNF binding activity (which required three N-terminal CRDs), only the first two CRDs appear to be required for the inhibition of apoptosis.

In myxoma virus infected cells, M-T2 is secreted into culture medium beginning at 2 h post-infection, providing extracellular M-T2 (71). However, the secretion of M-T2 becomes progressively more inefficient after 4 h p.i. and ceases by 12 h with nascent M-T2 being fully retained within the infected cells at later times (71). This suggests that continuous synthesis but inefficient secretion causes M-T2 to exhibit an intracellular localization, presumably within the endoplasmic reticulum or Golgi compartments. Pulse-chase analysis followed by immunoprecipitation with anti-T2 antibody detected two glycoforms of M-T2. Unlike most secreted viral proteins, a subset of M-T2 fails to undergo the standard terminal sugar modifications, in that most intracellular and some secreted M-T2 remained sensitive to digestion with Endoglycosidase H (Endo H) throughout the entire infection course (71). As most secreted proteins traffic through the Golgi stacks, terminal modifications to the glycosylation chain usually render the processed glycoprotein resistant to Endo H. Thus, the appearance of a pool of Endo H sensitive M-T2 leads us to speculate that some M-T2 is retained in a cellular compartment prior to the Golgi complex.

Current Models for the Dual Activities of M-T2

To date, several models have been proposed to explain the dual functions of M-T2 (55). First, M-T2, as a TNF-R homologue, is secreted from infected cells and binds directly to membrane-anchored or soluble TNF (Fig. 5b). In myxoma virus infected cells, it is estimated that M-T2 protein is produced in amounts in considerable molar excess over the cellular TNF receptors (71). Given the fact that M-T2 binds to TNF with an affinity comparable to that of cellular TNF-R,

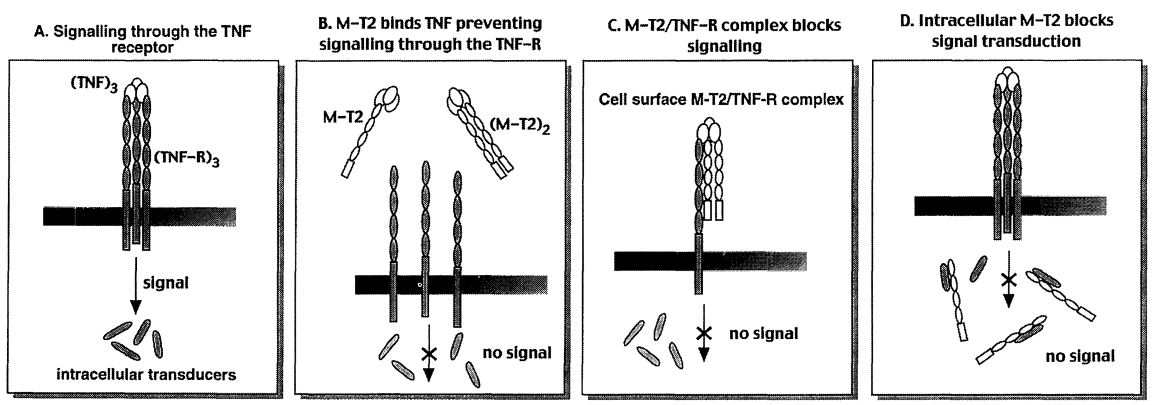

Fig. 5. Models to explain the inhibition of TNF and apoptosis by M-T2. Panel A, Signaling through the TNF receptor is initiated by clustering of TNF-receptors by trimeric TNF, thus allowing the activation of intracellular signal transduction molecules. Panel B, M-T2 monomers and dimers bind extracellular TNF, though only dimeric M-T2 is capable of effectively inhibiting signaling by the TNF trimer. Panel C, Hypothetical interaction between M-T2 and a TNF-receptor monomer in a dominant-negative fashion might act to block receptor oligomerization, and thus prevents signaling and apoptosis. This could also apply to other members of the TNF-receptor superfamily, potentially extending the action of M-T2 beyond inhibition of TNF responses. Panel D, Hypothetical association between the intracellular form of M-T2 and intracellular signal transduction molecules required for propagating the apoptotic signal.

it is likely that M-T2 is able to compete with the natural receptor and thereby inactivate extracellular TNF in a fashion reminiscent of shed cellular TNF-Rs (72,73). Similar mechanisms have been identified in the antagonistic decoy receptors for TRAIL and Fas ligand (41–43,74,75). For example, TRAIL-R3 uses the N-terminal ligand binding domain to interact with TRAIL, and thereby blocks the subsequent signal transduction since it lacks a cytoplasmic signalling domain (41–43). Based on the structural features of TNF-Rs, it is also possible that M-T2 might form a heterocomplex with membrane-bound cellular TNF receptors thus acting in a dominant-negative manner to interfere with further TNF-R oligomerization required for subsequent signaling (Fig. 5c). In terms of anti-apoptotic activity, only the first two CRDs of M-T2 are required to form dominant-negative complexes with TNF receptors, or possibly other superfamily members involved in apoptosis. Since the addition of extracellular M-T2 protein could not block apoptosis of lymphocytes infected with vMyxT2G, the formation of this heterocomplex could in theory occur in the endoplasmic reticulum prior to egress to the cell surface. By disrupting the proper conformation of nascent pro-apoptotic receptors, M-T2 would thus block programmed cell death signals mediated by their cognate ligands.

There is now persuasive evidence for an intracellular role of M-T2. Therefore, it is also theoretically possible that intracellular M-T2 might interact with one or more apoptosis or proliferation signalling molecules, and thereby inhibit the apoptosis cascade downstream of the TNF-R superfamily (Fig. 5d). We are now attempting to determine the exact localization of M-T2 and identify potential M-T2 binding partners that may help us understand the mechanism by which M-T2 exerts its anti-apoptotic function.

Conclusions and Perspectives

Poxviruses have proven to be a powerful model for understanding the virus-host interaction and co-evolution. So far, many viral proteins have been identified as virulence factors that play important roles in subverting immune responses. Amongst these, M-T2 of myxoma virus was initially characterized as a soluble TNF receptor homologue and shown to be capable of binding and sequestering TNF in a species specific fashion. It was subsequently discovered that M-T2 also acts intracellularly to block myxoma virus infected T cells from undergoing apoptosis. Although this latter pathway used by M-T2 has not been clarified yet, the anti-apoptotic function appears to be operationally distinct from the anti-TNF function in that overlapping but non-identical protein domains of M-T2 are required for each activity.

To date, certain virulence factors remain unique to viruses, and have no known cellular homologs. Nevertheless, it is possible that the cellular homologs of these ''orphan'' viral proteins will be uncovered as we learn more from other related fields. M-T2 is an example of a viromodulator in which one domain (the N-terminal CRDs) is clearly host-derived, while another domain (the non-homologous C-terminus) is unique. More myxoma virus encoded immuno-modulators will likely be identified as we complete the sequencing of the entire myxoma virus genome. It is our hope that these studies, combined with the current database of viral immuno-modulators will shed further light on the intricacies of the immune system.

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Poxvirus Homologues of Cellular Genes

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Abstract. Over the course of time poxviruses have acquired or ''captured'' numerous homologues of cellular genes and incorporated them into their large DNA genomes. With more poxvirus genome sequencing data becoming available, the number of newly discovered poxviral cellular homologues is constantly increasing. A common feature of these genes is that they are nonessential for virus replication *in vitro* and they confer selective advantages in dealing with host cell differentiation and immune defense mechanisms *in vivo*. Poxviral cellular homologues are reviewed in this synopsis considering the specific viral habitats of different poxviruses and the immune defence capabilities of their respective hosts. Possible mechanisms of cellular gene acquisition by poxviruses as suggested by the analysis of mobile genetic elements in large DNA viruses are discussed. The investigation of poxvirus homologues of cellular genes is essential for our understanding of the mechanisms that regulate virus/host interactions on the cellular level and the host response against infection.

Key words: poxviruses, cellular genes, pathogenicity, host, immunemodulation, cell differentiation

Introduction

Depending on the specific host organism and the primary or exclusive site of viral replication, different poxviruses evolved to employ host and tissue specific pathogenic strategies. The presence of cellular gene homologues in the genome of poxviruses reflects the underlying molecular mechanisms of this adaptation process with gene acquisition from and coevolution with the host as principal tools. The evolution of poxviruses dealing with increasingly complex mammalian immune systems resulted in the acquisition and development of viral homologues of cellular immunemodulatory genes involved in the host response to infection (1,2). Terms like ''viroceptors'' for soluble viral cytokine receptors (3) or ''cytokine response modifiers'' (crm) for the cowpox virus immunomodulatory genes (4,5) were introduced. Acquisition of cellular genes into viral genomes was also observed in other families of large DNA viruses, e.g. *Herpesviridae* and *Iridoviridae* (see Tidona and Darai; Raftery et al.; same issue) and is best described for retroviruses (6,7). Our knowledge of mammalian genes is rudimentary and one can assume that many of the large number of poxviral genes without known functions are of cellular origin. In particular, some of these genes are likely to have functions that are essential for virus replication and pathogenicity in the different hosts (8). The goal of this review is to give a summary of the most recent discoveries on cellular gene homologues in vertebrate poxviruses while emphasising the host and tissue specific nature of poxviral adaptation processes. Vertebrate poxviruses have been extensively studied and the most is known about the genetic makeup of their host organisms. Because our knowledge about the insect host is very limited, the large group of invertebrate (entomo-) poxviruses has been excluded. Within the vertebrate poxvirus family we can generally differentiate between poxviruses that cause acute, systemic and potentially lethal infections and those that cause benign, chronic infections that are confined to specific host tissues but are difficult to eradicate. The former include all orthopoxviruses as well as the lepori-and most other animal poxviruses, the latter

are the members of the genera molluscipoxvirus and parapoxvirus (9–12).

The term ''poxviral cellular gene homologue'' was defined as follows: viral genes of vertebrate poxviruses that have significant amino acid sequence homology to cellular proteins and/or highly conserved amino acid motifs but are not essential for replication in cell or tissue culture. The review is divided into three parts. The first part provides a summary of the most current genomic analysis of the family *Poxviridae*. The second part deals with the known poxviral homologues of cellular genes according to their tissue localisation and known functions. (Tables 1 and 2) The basic differentiation between extracellularly and intracellularly active poxviral homologues of cellular proteins has been extended by a class of molecules that are membrane associated. In the third part of the review examples are used to illustrate possible genetic mechanisms underlying the acquisition of cellular genes into poxvirus genomes.

DNA Primary Structure Data on the Family *Poxviridae*

The necessary basis for comparative studies on poxvirus homologues of cellular genes is extensive DNA nucleotide sequence data on poxviral genomes. Orthopoxviruses are the members of the family *Poxviridae* that are most thoroughly characterized by genomic DNA nucleotide sequence analysis. Of the orthopox prototype vaccinia virus (VV) many different strains were used for the eradication of smallpox (13–15). These include the vaccinia strains Copenhagen (COP) and Western Reserve (WR), the Lister (Elstree-WHO) strain, the Chinese strain Tian Tian, and the modified vaccinia virus Ankara (MVA). The complete primary structure of the genomes of two vaccinia virus strains, COP and MVA, was determined (16–18). In addition, DNA nucleotide sequences of many individual orthopoxvirus genes are now available. It is of importance to note that VV undergoes genetic variation when cultured *in vitro* or passaged on susceptible hosts for vaccine production. Genetic variation between the VV strains is best illustrated by the example of MVA. MVA is a VV strain that was attenuated by serial passage through chicken embryo fibroblasts (CEFs). The genome of MVA is with 178 kbp significantly smaller than that of the vaccinia COP genome (192 kbp). This is due to multiple large genomic deletions in comparison to VV strain COP. These genetic events are responsible for the restricted host range of MVA and alter MVA immunogenicity in comparison to VV (19–22). Deletions and truncations are common during extensive passaging/attenuation of VV.

Of the cowpox virus two strains are best characterized: Cowpox strain Brighton Red and GRI-90. From DNA sequence analysis of about 102 kbp flanking DNA sequences of GRI-90 (23) and comparison to the corresponding DNA regions of VV and variola strains it is known that about 20 kbp DNA sequence are unique to cowpox strain GRI90. The entire genome of cowpox virus has not been sequenced. The primary structure of the genomes of two variola strains, Bangladesh-1975, India-1967, has been completely determined by DNA nucleotide sequence analysis (24–26). On the amino acid level many variola proteins have either variola-specific sequences or divergent open reading frames compared with their VV counterparts. The necessary further investigation of these unique and variant variola virus proteins was the basis for the decision of the international virological community to preserve the last remaining variola strains rather than destroy them immediately (27–29). Monkeypox virus was recently associated with smallpox-like infections in Zaire (30). In contrast to past monkeypox episodes, where infections were almost exclusively transmitted from monkeys to humans (31,32), in the recent case reports it appears that more human to human transmissions occur (33,34). Recent molecular studies indicate that variola virus is ancestral to monkeypox virus, because of the presence of deletions in monkeypox virus genes that are intact in variola. This was interpreted that monkeypox virus should not be able to evolve into a variola type virus (35). Genomic sequence analysis of monkeypox virus isolates is needed to determine the genetic footprints of this phenotypic change. At the moment only very little genomic sequence data is available for a small number of monkeypox virus genes and isolates (36–38; unpublished GenBank submissions). Swinepox virus, the prototype virus of the genus *Suipoxvirus*, is mostly uncharacterized at the molecular level. The dsDNA genome is 175 kb in size, terminally cross-linked and contains terminal inverted repetitions (39,40). There is only very little DNA nucleotide sequence data on this virus.

The genus *Yatapoxvirus* includes tanapox virus, that causes vesicular skin lesions in humans. Other

members of the genus are yaba-like disease virus, that causes vesicular skin lesions in monkeys, and yaba monkey tumour virus, that causes epidermal histiocytoma. There is also very little DNA nucleotide sequence data on the members of this poxvirus genus.

Leporipoxvirus sequences were obtained early from the myxoma virus terminal inverted repeats and revealed several open reading frames that turned out to be important pathogenicity genes (41). A complete myxoma or Shope fibroma virus genomic sequence has so far not been reported. The natural hosts of parapoxviruses are various animals including camels, goats, cattle, deer, and sheep. Occasionally some of them cause zoonotic diseases in man, particularly Orf virus, pseudocowpox and bovine pustular stomatitis virus. Parapoxviruses contain linear dsDNA about 135 kbp in size with a G + C content of over 60%. The best-studied parapoxvirus is Orf virus. The epitheliotropic Orf virus induces acute pustular lesions in the skin of sheep, goats, and man and has a worldwide distribution. Extensive DNA nucleotide sequence data is not available. Molluscum contagiosum virus (MCV) is a member of the poxvirus family that exclusively replicates in the human epidermis. It causes benign cutaneous neoplasms in children and sexually active adults as well as persistent opportunistic infections in immunocompromised individuals. The DNA nucleotide sequence of the 190-kbp genome of MCV was recently determined (10,42–44).

Functional Gene Groups

Extracellular Proteins

TNF Receptor Homologues

Orthopoxviruses encode as many as three tumour necrosis factor (TNF) receptor homologues (vTNFR), all of which are secreted and able to bind various subsets of TNF ligands. The intense orthopoxviral coverage of immune molecules of this type reflects the major impact of the general inflammatory response induced by TNF ligands on orthopoxvirus infections in the host. vTNFRs were not found in the genomes of MCV and Orf virus. TNF may be the major cytokine involved in the sequestration and clearance of systemic orthopoxvirus infections (1,45–49).

Type 1 TNF Receptors

The inverted terminal repeats of cowpox virus strain Brighton Red contain two copies of the crmB gene (4,50). The crmB gene product is a 355-amino-acid protein expressed early during the infectious cycle, with a signal peptide sequence and three potential N-linked glycosylation sites. The protein is secreted from infected cells as an extracellular soluble protein with an apparent molecular mass of 48 kDa. TNFα and TNFβ bind to this protein in a competitive manner. The aminoterminal portion (176 amino acids) of the crmB gene product shares homology with the human cellular TNF receptor sequences. The C-terminal 161 amino acids of the crmB protein show homology only to the similar gene products of other poxviruses (23). Overall, the crmB protein is similar to the T2 proteins of the leporipoxviruses (51–53) and the predicted product of the G2R open reading frame of variola virus (4). The myxoma virus gene MT2 is a soluble TNF type 1-receptor that has an additional activity not found in its orthopoxvirus counterparts. The myxoma MT2 gene product prevents apoptosis as an independent function (48,49,54). A MT2 deletion mutant was attenuated in rabbits (3). An amino acid sequence homology between MT2 and the B28R and C22L open reading frames of VV COP was also found but both vaccinia genes are probably inactivated by frameshift mutations (17,18,55,56). All poxviral TNF type 1-receptor homologues described so far inhibit host TNFα and β activity.

Type 2 TNF Receptors

The cowpox virus strain Brighton Red contains another crm protein, crmC, that has amino acid sequence homology to the vaccinia protein A53 and belongs to the TNF type 2 receptor family (23,50). A crmC homologue or counterpart was not found in either myxoma or variola virus. CrmC is a single copy gene, and expressed late in the infectious cycle. It encodes a soluble, secreted protein of 186 amino acids with an apparent molecular weight of 25 kDa. The cysteine-rich recombinant protein binds TNF specifically and completely inhibits TNF-mediated cytolysis. The strongest sequence homologues are the ligand-binding regions of the type 2 cellular TNF receptor and crmB. The well conserved C-terminal portion (~ 150-amino acids) of crmB proteins is not found in crmC proteins. The function of crmC is viral

Table 1. Functional spectrum of secreted poxviral homologues of cellular genes

Tissue Localization	Cellular Function	Viral Homolog	Virus	Demonstrated/Predicted Function	Reference Numbers
Extracellular	TNF receptor	Crm B	cowpox	Type 1 early soluble TNF receptor; inhibits TNFα/β	4,23,50
		MT2	myxoma (2 copies)	action and apoptosis	51–53
		B28R/C22L	vaccinia (COP)		17,18,55,56
		G2R	variola		4
		Crm C	cowpox	Type 2 late soluble TNF receptor; inhibits TNFα	23,50
		A53R	vaccinia (COP)		17,18
		ND[a]	tanapox		57,58
		Crm D	cowpox	Type 3 late soluble TNF receptor; inhibits TNF and lymphotoxin-alpha (LTalpha)	59
	Interleukin	B15R/B16R	vaccinia (WR/COP)	Soluble IL1β receptor; inhibits IL1β action	68,69
	receptor	B15 homolog	cowpox		71,72
	Interleukin	mc053/54R	MCV	Soluble IL-18 binding protein; potentially inhibits IFNγ	43,78
	Receptor			and reduces NK cell activity	
	Interleukin	OV-NZ-2 IL 10	orf (NZ2)	IL-10 homolog	83
	homolog				
	Interferon	MT7	myxoma	Soluble INFγ receptor: inhibits INFγ action	90
	receptor	B8R	vaccinia (COP)	MT7: above and promiscuous CC and CXC chemokine	45,46,91–95
		B8R homolog	cowpox	binding	
		C6L	swinepox		
	Chemokine	B29R/C23L	vaccinia (COP,LIS)	Soluble chemokine binding protein; 35 K major secreted	100–103
	receptor	B29R homolog	cowpox	protein;	
		MT1	myxoma	2 copies; silent in WR	104
	Chemokine	mc148R	MCV	CC-chemokine antagonist	42–44,107,108
	homolog				
	Complement	C3L-VCP	vaccinia (COP)	Soluble C3B-C4B vaccinia complement binding protein	23,109–117
		IMP	cowpox	(VCP); inhibits inflammation; cowpox	
				immunemodulatory protein (IMP)	
	Growth factors	C11R	vaccinia (COP)	Vaccinia growth factor (VGF)	119–128
		ND[a]	myxoma	Myxoma growth factor (MGF)	56,129–134
		ND[a]	orf	Mammalian endothelial growth factor homolog	137,139

Table 2. Functional spectrum of membrane associated and intracellular poxviral homologues of cellular genes

Tissue Localization	Cellular Function	Viral Homolog	Virus	Demonstrated/Predicted Function	Reference Numbers
Membrane	Interferon receptor	B18R/B19R	vaccinia (COP/WR)	INF α/β receptor: reduces febrile response to infection (partially secreted)	69,146–149
		B18R homologue	cowpox		
	Complement	B5R	vaccinia (COP)	Complement like glycoprotein: EEV class I membrane glycoprotein: viral egress	151–157
		B17R	variola (INDIA)		
	SLAM	mc002L, mc161R, mc162R	MCV	SLAM homologs	43, 44, 160
	Chemokine receptor	K2R	swinepox	G-protein coupled receptor (predicted)	163
		ND[a]	capripox		164
	Apoposis inhibitor	M11L	myxoma	Membrane associated and secreted: inhibits apoptosis (also MT2, MT4, MT5)	54, 165–167, 173
Intracellular	PKR	K3L	vaccinia (COP)	Inhibits eIF2α phosphorylation: INF resistance	87, 89, 180–185
		E3L	vaccinia (COP)	PKR homolog: INF resistance	
		E3L homologue	orf		186, 188
	Caspase inhibitors	B13R/B14R	vaccinia (WR/COP)	Serpininhibitor (SPI-2); IL-1β-converting enzyme (ICE/caspase 1) and FLICE (caspase 8) inhibitor:	193–196
		Crm A	cowpox		
		Serp-2	myxoma	general caspase inhibitor	197–199
		mc159R, mc160R	MCV	DEAD domains-FLICE inhibition	43, 44, 175, 200
	Antioxidants	mc066L	MCV	Glutathione peroxidase homologues	43, 160, 202, 203
		O2L, O4L	Vaccinia (COP)		18, 204
	Steroid metabolism	A44L	vaccinia (COP)	Steriod dehydrogenase homologue	18, 68, 205–209
		mc152R	MCV		43, 44, 210
	Differentiation	mc0131	MCV	DNJ domain: host cell differentiation control	43, 44
	MHC homologs	mc080R	MCV	MHC class I heavy chain homolog	43, 44, 160, 223

inhibition of host-elicited TNFα. Another TNFα inhibiting soluble viral TNF receptor has been described for tanapox virus. Tanapox virus infected cells secrete an early 38 kDa glycoprotein that binds to human interferon-γ IFNγ, interleukin (IL)-2, and IL5 (57). The same glycoprotein of tanapox virus inhibits TNF-α-induced activation of the nuclear transcription factor-κ B (NFκB) and downregulates expression of E-selectin, VCAM-1 and ICAM-1 genes (58).

Type 3 TNF Receptors

Interestingly, cowpox virus strain Brighton Red contains a third TNF receptor-like gene, crmD, which encodes a 320-amino acid protein of 44% and 22% amino acid sequence identity to the two other cowpox TNF receptor-like proteins crmB and crmC, respectively (59). The crmD gene is truncated in three other cowpox strains and missing in a number of other orthopoxviruses. Four strains of ectromelia virus (mousepox) contain an intact crmD (97% amino acid sequence identity to cowpox crmD) but lack homologues to crmB and crmC. The disulphide-linked complexes of CrmD (250 kDa) are secreted by cowpox virus and ectromelia virus infected cells late after viral replication. Cowpox in contrast to ectromelia virus infected cells produce only small amounts of crmD. CrmD contains a signal peptide, a 151-amino acid cysteine-rich region, and C-terminal sequences with little amino acid sequence homology to the cellular TNFR C-terminal region that is required for signal transduction. The crmD cysteine-rich region binds TNF and lymphotoxin-α (LTα) and *in vitro* blocks their proinflammatory activity (59).

Interleukin Pathway

IL1 and IL18 both belong to the interleukin 1 family of cytokines. When injected intravenously into vertebrates, interleukin 1β (IL1 β) is a potent endogenous pyrogen. ProIL1β and proIL18 are both cut by the IL1β converting enzyme (ICE: caspase-1). In contrast to IL1β, IL18 is not an endogenous pyrogen. However, IL18 contributes to inflammation and fever because it is a potent inducer of TNF, chemokines, and IFNγ production and influences natural killer cell activity and expression of endothelial adhesion molecules (60–63). IL1 receptor type 1 (IL1RI) is the prototype of a family of proteins that share significant homology in their signaling domains, including *Drosophila* proteins, several plant proteins, and the human IL18/IL1Rrp (hIL18R;64). The high degree of amino acid sequence conservation between them indicates that the IL1 receptor is part of an ancient signalling system inducing generalized host responses against pathogens that cause systemic infections (65–67).

Interleukin Receptor Homologues

VV induces acute phase responses, e.g. fever, during systemic infections in vertebrate hosts. In this context, the finding that the B15R gene of VV strain WR (B16R in COP) encodes an abundant, secretory glycoprotein that functions as a soluble IL1β receptor (68,69) is of particular interest. The VV soluble IL1 receptor binds in contrast to its cellular counterparts only IL1β (human and mouse) but not IL1α or the natural competitor IL1 receptor antagonist. The expression of VV IL1β receptor in VV infected mice affects an important part of the systemic acute phase response to infection: The infected mice fail to develop a febrile response, which reduces the severity of the disease. Infection with a VV B15R deletion mutant accelerates the onset of symptoms and increases the mortality of mice infected intranasally (70). The soluble IL1β inhibitor is one of the few immunemodulatory genes that are intact and presumably functional in the highly attenuated VV strain MVA, that does not express soluble receptors for IFNγ, IFNα/β, TNF and CC chemokines (16). The B16R encoded IL1β inhibitor is not functional in VV strain COP, a more virulent virus than the widely used vaccine strains Wyeth, Lister, and MVA. Fever induced by the VV strain COP infection can be inhibited with IL1β specific antibodies. If the defective VV strain COP B16R is replaced with the active B15R gene of VV strain WR, infected animals do not develop a high fever and the disease is less severe. The IL1β binding activity is also present in the cowpox virus encoded B15R homologue (71,72). However, the respective variola virus gene is inactivated by frameshift mutations (25). This is an interesting example of how viruses that cause systemic infections neutralise systemic host responses that are detrimental to host survival and therefore shorten the duration of the viral infection. Consequently, infections with orthopoxviruses that through spontaneous genetic alterations are not able to

suppress host acute phase responses take a more severe clinical course. Furthermore, analogs of soluble IL1β receptors have not been detected in the genomes of poxviruses that do not cause systemic infections, e.g. MCV and Orf virus. Finally, Smith and co-workers make the more general conclusion that though many cytokines, e.g. IL1α, TNF, IL6 and IFNγ, are involved in systemic acute phase response to infection, IL1β may be most important for the mediation of fever in the vertebrate host (73).

MCV IL18 Binding Protein

IL18, the former IFN-γ inducing factor (IGIF), and IL12 (74–76) are secreted by macrophages and specifically human dendritic cells, e.g. Langerhans cells in human epidermis (77). IL18/IL12 expression in virus infected cells increases natural killer cell cytotoxicity, up-regulates ICAM-1 type adhesins important for immune cell diapedesis into tissues, and induces IFNγ expression in IL18 and IL12 receptor carrying T-lymphocytes (61–63). Two putative early MCV proteins, mc053R and mc054R, share significant amino acid sequence homology to the human IL18/IL1γ receptor IL1Rrp (hIL18R:78). This finding is of great interest because of its implications for tissue specific immune mechanisms. IL12 and IL18 may be the predominant type of IL1 like cytokines in the MCV site of replication, the human epidermis, and therefore need to be neutralised by MCV gene products. As shown in a later section of this review, another MCV protein, the Molluscum chemokine homologue, antagonizes other host cytokines, that control the movement of leukocytes in and out of the MCV infected epidermis. In a highly tissue adapted strategy MCV encodes at least three gene products that are involved in the suppression of the local host IFNγ response, natural killer cell activity, and infiltration of immune cells. The histological data confirm that this MCV strategy is very successful (79,80).

Orf Virus Interleukin Homologue

The first two examples of IL10-like cytokines expressed by viruses were discovered in members of the herpesvirus family: Epstein-Barr virus encodes BCRF1, a homologue of human IL10 that is expressed late during the viral infectious cycle (81). Equine herpes virus type 2 strain T400 encodes an open reading frame with significant amino acid sequence homology to the cytokine synthesis inhibitory factor (CSIF: IL10) of human (76.4%), mouse (68.5%), and to the Epstein-Barr protein BCRF1 (70.6%) (82). Recently, an IL10 homologue was found to be encoded by the *parapoxvirus* Orf virus (83). The amino acid sequence homology of the Orf virus (strain NZ2) IL10 homologue is closest to IL10 of sheep (80–100% in the C-terminal region), suggesting that the gene has been captured from its sheep host during the evolution of Orf virus. The Orf virus IL10-like gene is transcribed early. The gene product has IL10 specific activity and is secreted from infected cells (83). When Orf virus IL10 was expressed by VV in immunodeficient mice, natural killer cell activity was increased and virus replication was diminished compared to controls (84). However, this is an highly artificial system to test the *in vivo* role of Orf virus-IL10. IL10 is also known as cytokine synthesis inhibitory factor. Therefore, downregulation of proinflammatory cytokines in the human epidermis might be an important role of the Orf virus IL10 homologue *in vivo* (85,86) for more details see Mercer et al.; same issue).

Interferon Receptor Homologues

The first experiments to demonstrate interferon (IFN) resistance of vaccinia virus involved a coinfection of VV and vesicular stomatitis virus (VSV), a mammalian virus that is especially sensitive to the effects of IFN. VV protected VSV from the antiviral effects of IFN (87,88). These observations led to the discovery of the RNA dependent protein kinase (PKR)-associated VV interferon resistance mechanism (89).

Independently, a different mechanism of interferon resistance was described for leporipoxviruses (90), where the myxoma MT7 gene product was found to be a soluble IFNγ receptor (IFNγR). Consequently, it became clear that orthopox- and suipox viruses secrete a similar protein for inhibition of IFNγ effects in an PKR independent pathway (45,46,91–95).

The VV soluble IFNγR (VV strain WR B8R), is produced early during infection and efficiently blocks the binding of IFNγ to cellular receptors, negating the cellular IFN response. The gene product of B8R shares amino acid sequence homology with the extracellular binding domain of cellular IFNγR and is highly conserved among members of the genus

orthopoxvirus. Orthopoxvirus IFNγR bind and inhibit the biological activity of human, bovine, rat, and chicken (96), but not mouse IFNγ. The binding specificities of orthopoxvirus IFNγR reflect the coevolution of orthopoxviruses within their natural hosts as well as the hosts that were selected for the passaging of vaccine strains (45,46,94). In contrast, the IFNγR encoded by myxoma virus shows a less extensive amino acid sequence homology to cellular and orthopoxviral IFNγR. Interestingly, like cellular IFNγR, that are highly specific for the interferons produced within the same species, the myxoma virus MT7 IFNγR is highly specific for the IFNγ of its rabbit host. Furthermore, the myxoma virus MT7 gene product displays an additional activity that has not been observed in orthopoxvirus IFNγR. It interacts promiscuously with members of the CXC, CC, and C chemokine families (93).

Chemokine Antagonists

Chemokines are a family of small peptides (~ 100 amino acid residues) that direct migration of immune cells into sites of tissue injury. Chemokine signal transduction is mediated by chemokine receptors. Chemokine receptors are large, complex seven-transmembrane domain proteins (2,97–99). By their size and spatial structure chemokine receptors are not naturally suited for a viral anti-inflammatory strategy analogous to the secreted IFN receptors discussed in the previous sections of this review. Small viral chemokine binding proteins and chemokine homologues that can act as competitive antagonists to chemokine receptors are a natural alternative for inhibition of the local inflammatory host response.

Chemokine Binding Proteins

Infection of tissue culture cells with VV results in the specific secretion of several polypeptides into the medium. Infection with the Lister (100) and Evans strains of VV as well as other orthopoxviruses, e.g. rabbitpox, cowpox virus (101), and variola virus, results in the production of a protein with an apparent molecular weight of 35 kDa, that is secreted in large amounts at both early and late times during infection. This protein is not essential for growth in tissue culture (100). Surprisingly, a 35 kDa protein is not secreted by the WR, Wyeth or Tian Tan strains of VV. The gene encoding the Lister strain 35 kDa protein was mapped within the inverted terminal repeats of the genome (101). The DNA sequence of this region showed that the ends of this gene are very similar to the flanking sequences of a WR gene that encodes a protein with an apparent molecular weight of 7.5 kDa. In the vaccinia strain COP, this gene is identical to the very last open reading frames C23L/B29R on the 5′ and 3′ flanks of the genome, within the terminal inverted repeats, and therefore present in two copies. The nonsecreted 7.5 kDa polypeptide of WR is probably the result of a deletion event. Recently it was found, that the secreted 35 kDa protein binds and sequesters CC (β), but not CXC (α) or C (γ) chemokines with high affinity and therefore is a viral chemokine binding protein (102,103).

The homologue of VV C23L/B29R in myxoma virus is the product of the myxoma virus gene MT1. The 35–40 kDa secreted myxoma virus protein shares only about 40% amino acid sequence homology in comparison to the 35 kDa secreted protein of orthopoxviruses (104). The CC-chemokine binding and inhibitory properties of leporipoxvirus T1 and orthopoxvirus 35 kDa proteins do not appear to be species specific.

Surprisingly, another myxoma virus protein, the myxoma MT7 gene product (90), was first described to be an active soluble IFNγR homologue (92), but later was found to be a chemokine binding protein, too. It interacts with members of the CXC, CC, and C chemokine families (93,105,106). The viral chemokine binding proteins have no amino acid sequence similarity to known cellular chemokine receptors, all of them multiple membrane-spanning proteins. This could be interpreted that either an unknown cellular chemokine receptor of a soluble type exists or, alternatively, the viral chemokine binding proteins have no cellular homologue and were independently developed by host directed evolution in the poxvirus family (103). The anticipated function of chemokine binding proteins is inhibition of the proinflammatory (antiviral) activities of chemokines.

MCV Chemokine Antagonist

Clinical lesions of MCV are conspicuous for the absence of an inflammatory infiltrate (79,80). MCV lesions persist for months and are only cleared when an inflammatory response is induced by mechanical irritation of the lesion. The predicted activity of the

gene product encoded by MCV-1 open reading frame mc148R presents one possible explanation for the absence of inflammatory activity in early MCV lesions (43). The 104 amino acid protein encoded by mc148R has structural similarity to the β (CC) family of chemokines. A predicted signal peptide is followed by four cysteine residues, of which the first two are direct neighbors (CC). However, a 5 amino acid deletion in the hypothetical N-terminal activation domain of this MCV chemokine homologue (MCCH) suggested the absence of chemoattractant activity (43). The amino acid sequence of MCCH was found to be highly conserved in independent isolates of MCV type 1 and 2. MCCH transcripts were detected *in vivo* in MCV infected tissue specimen (42). When MCCH was expressed in a baculovirus system and analyzed in leukocyte migration assays it showed no chemotactic activity but blocked the chemotactic response to macrophage inflammatory protein (MIP)-1α, a human β-chemokine. Baculovirus expressed MCCH furthermore inhibited the growth of human hematopoietic progenitor cells *in vitro* (107). Using a VV expression system, the recombinant MCCH polypeptide was expressed in mammalian cells with an apparent molecular weight of 10 kDa and secreted into the medium (42). Recombinant MCCH expressed in the VV system interfered with the chemotaxis of human monocytes, lymphocytes, and neutrophils in response to the CC chemokines MCP1, MCP3, MIP1α, RANTES and 1309 and to the CXC chemokines IL8 and SDF1 (108). The ability of the MCV chemokine antagonist to block the action of both CC and CXC chemokines and the presence of two genes encoding putative IL18 binding proteins underlines the significance of local inflammation control for the maintenance of the early stages of MCV infection in human skin.

Complement Control Proteins

Members of the family of complement control proteins inhibit complement-mediated opsonisation of bacteria and induction of inflammatory and phagocytic responses *in vitro* (109,110). The VV complement control protein (VCP) was the first soluble microbial protein to have a postulated role in the immunemodulation and evasion of host defence and was subsequently found in cowpox virus (23,111,112).

VV strain WR open reading frame C3L encodes a 35 kDa major secretory polypeptide VCP which is structurally related to the family of human and mouse complement control proteins (113,114). VCP binds to human C3 and C4 and blocks the complement cascade at multiple sites (115). The *in vivo* role of this protein was studied in rabbits, guinea pigs, and mice using vaccinia C3L deletion mutant viruses and C5 deficient mouse strains (116). In these animal models the presence of complement control proteins or absence of complement activity diminishes destruction of virus infected host tissue, thus preserving the viral habitat (117).

Growth Factors

Orthopoxvirus *Growth Factors*

Epidermal growth factor (EGF) and transforming growth factor type I (TGFα) bind to EGF receptor. This evident leads to the phosphorylation of the EGF receptor, stimulation of its kinase activity and cell growth (118). The early VV gene C11R encodes a small polypeptide of 140 amino acid residues with an apparent molecular weight of 19 to 25 kDa and sequence homology to EGF and transforming TGFα (119,120). The homology particularly involves six positionally conserved cysteine residues that form disulphide bond mediated loop structures (121). The viral EGF homologue is present in two copies within the inverted terminal repeats of VV strain WR (122), but only one is found in the VV strain COP genome (18). A VV C11R-vEGF deletion mutant replicates well *in vitro*. However, it replicates to higher titers in growing cells than in resting cells (122). The presence of signal peptide and transmembranous sequences indicates that a membrane-associated form may be the precursor of a soluble growth factor. The glycosylated 25 kDa vaccinia polypeptide is secreted, competes with EGF for binding to the EGF receptor and is a potent mitogen (123–125). A nonglycosylated form of VGF is a cell growth inhibitor (126). *In vivo*, higher doses of VGF-deletion mutant virus than wildtype VV virus are required for intracranial lethality in mice and for production of skin lesions in rabbits. Thus, expression of the VGF gene is important to the virulence of VV. Viral production of EGF-like growth factors is a possible explanation for the proliferative nature of diseases caused by SFV, Yaba tumor virus,

and certain orthopoxviruses (122). Despite earlier reports (127), the human epidermal growth factor receptor is not the cellular receptor necessary for entry of VV into its host cell (128).

Leporipoxvirus *Growth Factors*

Myxoma virus and Shope fibroma virus both possess genes with the potential to encode EGF-like polypeptides (56,129). Myxoma growth factor (MGF) and Shope fibroma growth factor (SFGF), are both 35 kDa polypeptides, are expressed early in infection and compete with EGF for its receptor (130). Myxoma virus growth factor deletion mutants induce less proliferation of the epithelial cell layers in the conjunctiva and respiratory tract and are significantly attenuated in the rabbit host (131). In a recombination event that occurred in wildlife, the myxoma growth factor gene was deleted and completely replaced by the Shope fibroma virus gene. This event led to new phenotype, malignant rabbit virus (MRV; malignant rabbit fibroma virus. MRFV), with increased virulence in the wildliving rabbit hosts (132). SFGF is a major virulence factor in MRV infection and is responsible for at least some of the cellular proliferation observed at tumour sites (133). The growth factors of Shope fibroma virus, myxoma virus and VV display unique patterns of specificity to ErbB receptor tyrosine kinases. SFGF is a broad-specificity ligand, VGF binds primarily to ErbB-1 homodimers, and the exclusive receptor for MGF is a heterodimer comprised of ErbB-2 and ErbB-3. Compared to their mammalian counterparts the viral ligands have 1 to 2 magnitudes lower binding affinity but have about the same mitogenic activity. This was found to be due to reduced receptor degradation, leading to sustained signal transduction despite low-affinity ligand-receptor interaction (134).

Orf Endothelial Growth Factor

Mammalian vascular endothelial growth factor (VEGF) mediates endothelial cell proliferation, angiogenesis, and vascular permeability via the endothelial cell tyrosine kinase receptors KDR/Flk-1 (VEGFR2) and Flt-1 (VEGFR1). VEGF binds and activates these two receptors and requires neuropilin-1 and 2 as coreceptors. It has been proposed that VEGFR1 mediates cell migration whereas VEGFR2 mediates cell proliferation (135,136).

Two copies of an Orf virus homologue of vascular endothelial growth factor (VEGF) are encoded outside the inverted terminal repeat at the right end of the genomes (137). The genes are transcribed early during infection. The VEGF homologue in the NZ2 strain of Orf virus is a polypeptide with an apparent molecular weight of 14.7 kDa. The size of the protein encoded by a variant gene in the NZ7 strain of Orf virus is 16 kDa. The Orf virus NZ2 and NZ7 polypeptides show 22% and 16% identity, respectively, to the mammalian VEGFs. The viral polypeptides share only 41.1% amino acid sequence identity, and there is little homology between the two genes at the nucleotide level. Both Orf gene products carry the characteristic cysteine knot motif found in all mammalian VEGFs and many other growth factors (138) but form a group distinct from previously described members of this family. The VEGF genes of Orf virus have a low G + C content in comparison to the G + C content of the viral genome. The G + C content of the Orf genome is 63% whereas the Orf NZ2 gene G + C content is 57.2% and the Orf NZ7 gene G + C content is 39.7%. Of the two only the Orf NZ2 gene is homologous to the mammalian VEGF genes at the DNA level. This may be an indication that the two Orf genes have been acquired from the mammalian host, the NZ2 gene more recently than the NZ7 gene.

Receptor binding characteristics of the Orf NZ7 derived VEGF were analyzed and compared with mammalian VEGF (139). Orf NZ7 VEGF shows almost equal levels of mitotic activity on primary endothelial cells and vascular permeability activity compared to mammalian VEGF. Orf NZ7 VEGF binds and activates the VEGFR2 but does not bind to the VEGFR1. The Orf NZ7 VEGF seems to be a novel type of endothelial growth factor that activates only VEGFR2 and induces a potent mitogenic and angiogenic activity (139).

MCV Growth Factors

The presence of a growth factor homologue in MCV was initially suspected from the proliferative nature of the MCV lesion (120). However, despite early attempts to identify a MCV EGF-like growth factor homologue (140) the analysis of the complete genome sequence of MCV did not give any indication of known growth factor-like gene products. However,

some putative secreted MCV proteins may have growth factor-like activities (43).

Membrane Proteins

The most extensive research on poxviral membrane proteins was done on VV. During maturation VV takes advantage of the host cell membrane and lipid metabolism. VV virions are processed through the Golgi and eventually end up fully matured as extracellular virus particles with three lipid membrane layers (141,142). VV produces two types of infectious virus particles: intracellular mature virus (IMV) and extracellular enveloped virus (EEV). EEV in comparison to IMV carries an additional lipid membrane (from the Golgi) and at least ten EEV proteins, that are not all poxvirus-encoded and that are absent from IMV. EEV represents less than 1% of infectious progeny. Nevertheless, it mediates virus dissemination and is critical for cell-to-cell and long-range spread of the virus. EEV is the virus against which protective immune responses are directed. Furthermore its cellular receptors are different from the ones for IMV (143–145).

Membrane Associated Interferon Receptor

VV and other orthopoxviruses, including MVA (16), express a both soluble and membrane associated type I interferon (IFN) receptor. In VV strain WR the gene B18R (COP B19R) encodes a glycosylated protein of 60–65 kDa that has regions of significant amino acid sequence homology to the α subunits of the mouse, human, and bovine type I IFN receptors (69,146). The membrane-associated form of the protein is part of the EEV (145). The VV strain WR B18R encoded receptor has high affinity for human IFN α, but like the soluble interferon receptor (VV strain WR gene B8R) has broad species specificity, binding to human, rabbit, bovine, rat, and mouse type I IFNs. VV B18R deletion mutants have an attenuated phenotype in mice (147). The B18R gene product inhibits the binding of the type I cellular interferons (IFN)-α, -β, -δ and -ω of different mammalian species (148,149). The VV-WR B18 R gene product is both a soluble extracellular and a cell surface protein. This is an indication that it should be able to block both autocrine and paracrine functions of IFN (146).

Membrane Associated Complement Homologue

EEV in contrast to IMV is resistant to neutralisation by antibody (145). It is also resistant against complement activity both in the presence and absence of specific antibodies (150). At least six poxviral genes encode EEV-specific proteins (151). One of them, the VV-WR gene B5R, encodes a 42 kDa glycoprotein (gp42) and is transcribed both early and late during infection. The protein is present on EEV but not on IMV membranes. The B5R gene product has significant amino acid sequence homology to members of the complement control protein superfamily, including VV C3L-VCP. The extracellular/secreted portion contains four copies of a 50- to 70-amino-acid short consensus repeat (SCR) typical for proteins of the complement control superfamily. The B5R gene product differs from C3L-VCP in so far as it contains a C-terminal transmembrane domain in addition to a signal peptide sequence and therefore may be membrane-associated as well as secretory. The gp42 protein forms 85 kDa hetero- or homodimers under nonreducing conditions (152,153). Deletion of the B5R gene from the genome of VV results in a 10-fold reduction of EEV, and as a consequence, plaque morphology changes to a small-plaque phenotype *in vitro*. The B5R deletion mutant virus was found to be attenuated *in vivo* (151,154,155). The B5R protein is probably a viral keyprotein in the process of VV assembly (156) and is less likely linked to immuneevasion (157). This is supported by the fact that neither the B5R complement-like glycoprotein nor any other poxvirus encoded proteins are required for the observed complement resistance of EEV. EEV was found to be resistant to complement only when the virus was grown in cells of the same species. As suggested by Smith and co-workers, VV complement resistance is mediated by host complement control proteins incorporated into the outer envelope of poxviral EEVs (150). Similar complement evasion strategies were reported for human T-cell lymphotropic virus type 1 and human cytomegalovirus (158).

MCV-Signaling Lymphocyte Activation Molecule

One of the marker proteins rapidly induced on naive T cells and B cells following activation is the signaling

lymphocyte activation molecule (SLAM; 159). SLAM is a multifunctional 70 kDa glycoprotein member of the immunoglobulin superfamily. Known SLAM functions are IL2-independent expansion of activated T cells during immune responses, induction and/or up-regulation of IFNγ by activated T cells, and differentiation of these proliferating cells to Th0/Th1 phenotypes. SLAM exists in a secreted and a membrane associated form and is a high affinity self-ligand. The MCV gene family mc002L, mc161R, and mc162 has significant amino acid sequence homology to cellular SLAM (43,44). The MCV SLAM family of proteins are all transcribed early in infection (160; unpublished observations). Neutralisation of soluble cellular SLAM by membrane associated MCV SLAM homologues on MCV infected cells and/or competitive binding of secreted MCV SLAM homologues to cellular SLAM on activated B- and T- cells are possible mechanisms of action for the MCV viral SLAM homologues.

G Protein-Coupled Receptors

G protein-coupled receptors transduce extracellular signals that modulate the activity of a wide variety of biological processes, such as neurotransmission, chemoattraction, cardiac function, olfaction, and vision, involving intracellular responses ranging from regulation of intracellular levels of cAMP to stimulation of gene transcription (161,162). Limited DNA nucleotide sequence analyzes of the genome of swinepox virus (SPV) revealed that open reading frame K2R encodes a putative protein with structural characteristics and amino acid sequence homology to the G-protein coupled receptor superfamily (163). Another putative protein of this type was found by DNA nucleotide sequence analysis of the capripox virus (KS-1 strain) genome near the left terminus. The partial open reading frame Q2/3L has significant amino acid sequence homology to members of the G protein coupled chemokine receptor subfamily, the swinepox virus K2R gene product and the human cytomegalo virus protein encoded by open reading frame US28. All members of the *Capripoxvirus* genus contain copies of this putative G protein coupled chemokine receptor homologue (164). These are the first examples for G protein-coupled receptor-like proteins encoded by poxviruses.

Membrane Associated Apoptosis Inhibitor

The product of the myxoma virus M11L gene is expressed early in infection (173). The sequence has a single transmembrane helix near the C-terminus and a N-terminal extracellular domain that has six cysteine residues plus two consensus N-glycosylation sites. The myxoma M11L gene product is transported to the membrane. Myxoma virus M11L deletion mutants are unable to cause the characteristic lethal disease symptoms of myxomatosis. If the M11L gene product is not present, a more vigorous inflammatory reaction with a higher influx of inflammatory leukocytes into the site of myxoma virus replication can be observed (165). M11L is a membrane-associated viroreceptor that recognises an as yet unidentified extracellular ligand essential for the cellular inflammatory response (166).

Infection of CD4+ T lymphoma cell line RL-5 cells with Shope fibroma virus or attenuated myxoma virus mutants containing a disrupted M11L gene leads to DNA fragmentation and general morphological changes characteristic for cell death by apoptosis (54). The same effect was observed when myxoma MT2, MT4, and MT5 deletion mutant viruses were used to infect RL-5 cells (54,167). McFadden and co-workers conclude that the myxoma virus genes M11L, MT2, MT4, and MT5 encode proteins with multiple activities. One of them is to extend the myxoma virus host range for replication in rabbit T lymphocytes through the inhibition of apoptosis (54,167).

Intracellular Proteins

During viral infections the intricate mechanisms of intracellular homeostasis are manipulated to promote viral replication by shutting down cellular mechanisms of translational and growth control. In the poxvirus field the earliest example for such a mechanism is the E3L/K3L mediated inhibition of RNA dependent protein kinase (PKR) to override cellular control of protein synthesis (168). More recently, new insights into programmed cell death (apoptosis) led to the discovery of a whole new class of viral homologues of cellular genes that interfere with these mechanisms (169). Some of them were previously identified as agents of poxviral immune evasion, like the serpin family of interleukin inhibitors (170,171). Others are hitherto unknown poxviral proteins, as for instance the MCV gene family of

Fas associated death-domain-like IL1β converting enzyme (FLICE: caspase 8) inhibitors (172–175).

At last, the modifiers of lipid and steroid metabolism and poxviral proteins that interfere with mechanisms of cellular differentiation belong to the group of intracellularly active poxviral homologues of cellular genes but are not obviously apoptosis related.

Protein Kinase R (PKR) Inhibitors

The interferon-inducible, double-stranded (ds) RNA-triggered protein kinase (PKR) regulates protein synthesis initiation by phosphorylating the α-subunit of eukaryotic translation initiation factor 2 (eIF2). The amino-terminal region of PKR contains two dsRNA-binding domains. The kinase domain is located in the C-terminal region of the protein. PKR is a ribosome-associated protein that has been attributed an important role in the intracellular inhibition of viral protein synthesis as well as in the control of cell proliferation (176–178). Human PKR (p68) functions as tumour suppressor gene by induction of apoptosis (179).

VV is relatively resistant to the antiviral effects of interferon-α (IFNα) and is able to rescue replication of IFN-sensitive viruses, such as encephalomyocarditis virus and vesicular stomatitis virus, from the antiviral effects of IFN (87). VV employs several independent mechanisms to counteract the interferon-induced antiviral host cell response. As shown above, soluble and membrane associated interferon receptors encoded by the vaccinia genes B8R and B19R (COP) neutralize the ligand by competitive binding. The products of two other VV genes, K3L and E3L, are active on the intracellular level. They cause the IFN-resistant phenotype of VV by interference with the activity of the double-stranded RNA-dependent protein kinase (PKR) (89,180–182). Both proteins are transcribed early during VV infection and expression can be observed in virus-infected cells as early as 0.5 hr postinfection. The E3L gene product inhibits PKR activity approximately 50- to 100-fold more efficiently than the K3L gene product. The two inhibitors act in different ways. The E3L gene product is a dsRNA binding protein and interferes with the binding of PKR to double-stranded RNA. The K3L gene product competes with eIF2α for its interaction with PKR, reducing the level of phosphorylated eIF2α in VV-infected cells. In a follow-up of the original observations, it was demonstrated that E3L rescues vesicular stomatitis virus from the effects of IFN and that K3L does at least partly the same for encephalomyocarditis virus (83,183,184). The product of VV gene E3L furthermore acts as a direct inhibitor of the IFN-induced 2–5A-synthetase enzyme (185). The fact that poxviruses not only reduce the amount of free IFN ligand using secreted and membrane bound IFN receptors, but furthermore have developed two independent ways to modulate the intracellular interferon signal transduction pathway underlines the importance of the IFNγ mediated type of cellular immunity.

Orf Virus PKR Inhibitor

Orf virus harbors an open reading frame (OV20.0L) which shares 31% amino acid sequence identity (57% similarity) to the VV interferon resistance gene E3L (186,187). The Orf virus gene is located 20 kbp from the left terminus of the Orf virus genome and is expressed early in infection and nonessential for replication *in vitro*. Its amino acid sequence contains four of the six residues identified as being essential to dsRNA binding in the VV protein. The Orf virus protein OV20.0L binds double-stranded (ds) RNA but not dsDNA, single-stranded (ss) DNA or ssRNA (186–188). The OV20.0L gene product inhibits interferon like the VV E3L gene product via the PKR pathway.

Poxviral Caspase Inhibitors and Apoptosis

The activation of cell surface receptors by extracellular proteases is the first step in the chain of events leading to apoptosis (189). Conformational changes of the cell surface receptors activate numerous intracellular proteases, most of which are cysteine requiring aspartate proteases (caspases; 190). Under these conditions control of proteolysis is essential for intracellular homeostasis. Apoptosis can be inhibited or promoted at many points of the intracellular signal transduction chain by specific protease inhibitors, e.g. members of the serpin family, some of which are encoded by viruses (169,191). Protease inhibitors that control activated proteases include the viral proteins crmA/SPI-2, the cellular granzyme B inhibitor PI-9, the extracellularly active cellular serpin PN-1 and the cellular serpin PAI-2 that targets both extracellular and intracellular proteases. Investigation of the functional properties of the poxvirus serpin crmA/SPI-2 has contributed significantly to our knowledge about proteolysis within apoptotic cells (192).

Poxvirus Homologues of Serpin Family Protease Inhibitors

VV encodes a 38.5 kDa intracellular polypeptide (WR B13R / COP B14R gene) that is non-essential for virus replication *in vitro* and does not affect virulence in a murine intranasal model (193). The B13R gene product belongs to the serpin superfamily, has 92% amino acid sequence identity with the cowpox cytokine response modifier A (crmA) protein and inhibits the IL1β converting enzyme (ICE-caspase-1). However, in contrast to the vaccinia IL1β receptor (B15R WR gene), it is not able to prevent fever in infected mice. Instead, the B13R protein blocks apoptosis induced by anti-Fas antibodies or by tumour necrosis factor (TNF) and cycloheximide (194,195).

The ability of cytolytic cells to cause apoptosis in target cells is in part mediated by the extracellular serine protease granzyme B. Granzyme B is inhibited by the cowpox viral serpin cytokine response modifier A (crmA) and presumably by its related proteins encoded by other poxviruses. The crmA related proteins have the unusual ability to efficiently inhibit proteases from two distinct catalytic classes, in this case serine and cysteine proteases and from two different compartments, intracellular ICE and extracellular granzyme B (196). Other members of the serpin/crmA superfamily (SERP-2) are encoded by myxoma virus and rabbitpox virus (197–199). As originally described, crmA prevents cytokine processing by inhibiting caspase-1. However, crmA is also an inhibitor of a number of other caspases including caspase 8 (FLICE) and protects against Fas-, TNF- and TRAIL- mediated apoptosis (172,173).

MCV Apoptosis Inhibition via the FLICE Pathway

MCV is another member of the poxvirus family that employs caspase 8 inhibition as a viral antiapoptotic strategy (44,200). MCV proteins mc159 and mc160 and the equine herpesvirus 2 protein E8 share substantial homology to the death effector domain present in the adaptor molecule Fas-associated death domain protein (FADD) and the initiating death protease FADD-like interleukin-1β-converting enzyme (FLICE: caspase-8). The viral proteins protect cells from Fas- and TNFR1-induced apoptosis. FLICE-induced apoptosis was not inhibited by either of the two proteins. It was concluded that the inhibitory action occurs upstream of the apoptosis effector FLICE and that MCV and EHV-2 regulate Fas- and TNFR1-mediated apoptosis using a novel control point (175).

Antioxidants

Investigation of the intracellular activities of the cowpox protein crmA and related proteins has also demonstrated that there are separable effector mechanisms within cells, and that those triggered by growth factor withdrawal, matrix dissociation, or cytotoxic ligands are different in several respects to those triggered by radiation, chemicals, or steroid hormones. Epidermal cells undergo a differentiation process on their way through the different layers of human epidermis that ends in UV induced apoptosis. This caspase mediated apoptosis causes major structural cell changes in the stratum corneum, characterized by the reorganisation of keratin 18 intermediate filaments into the typical granular structures (201). It is a likely scenario that poxviruses that replicate in epidermal cells will develop antiapoptotic mechanisms in order to extend their replication time.

Indeed it was found that MCV, a poxvirus that exclusively replicates in the human epidermis, uses a glutathione peroxidase homologue (mc066L;43,44) in a TNFR/Fas unrelated antiapoptotic strategy to block apoptosis resulting from reactive oxygen species induced by UV irradiation (202). Like the cellular glutathione peroxidase (GSHPx-1), the MCV enzyme has antioxidant activity, and requires the essential trace element selenium for its major function, the degradation of UV or oxygen burst induced free oxygen radicals that are associated with cellular damage (202). The MCV selenoprotein is transcribed early (160) and by virtue of a late conserved promoter signal probably also late in infection and protects human keratinocytes against apoptosis induced by UV irradiation and/or free oxygen radicals (203). The MCV mc066L gene is highly homologous to the cellular glutathione peroxidase gene on the DNA level, suggesting that the gene has been captured from the cellular genome and now undergoes genetic drift.

Interestingly, the O2L and G4L genes of VV (18) also encode two functional glutaredoxins. The product of the O2L gene is synthesized late in infection. The other vaccinia gene, G4L, encodes a glycosylated protein that has sequence similarity to

glutaredoxins, possesses thioltransferase and dehydroascorbate reductase activities (204) and is expressed early in infection. The protein encoded by G4L is homologous to MCV$\sqrt{\text{mc059L}}$, whereas the amino acid sequence of the O2L protein is not. Glutaredoxin activity of G4L-like enzymes may be beneficial for replication of poxviruses *in vivo*.

Modifiers of Steroid Metabolism

Mammalian 3-β-hydroxysteroid dehydrogenase (3-β-HSD) and plant dihydroflavonol reductases are descended from a common ancestor. The VV open reading frame A44L has 31% amino acid sequence identity to 3-β-HSD and dihydroflavonol reductase. The iridovirus fish lymphocystis disease virus (LCDV) open reading frame 153L shows a similar homology and both viral genes are most closely related to the mammalian 3-β-HSD (205,206). The 3-β-HSD encoded by the A44L gene of VV (68,207) is active *in vitro* (208) and can be detected in CV-1 cell cultures infected by different orthopoxviruses. Deletion of the gene in a VV mutant showed that the absence of the gene and the virally induced 3-β-HSD activity from infected cultures has no effect on viral replication *in vitro*. Intranasal infection of mice showed a slight attenuation of the virus mutant *in vivo* (208,209). In the genome of MCV open reading frame mc152R encodes a hypothetical protein that has 43.4% amino acid sequence identity to the VV A44L and 32.6% to the human gene (43,210). The function of these viral enzymes in virus infected cells is still unclear.

Differentiation

One of the most interesting MCV cellular homologues is the hypothetical protein encoded by MCV open reading frame mc013L because of its unique approach to host cell regulation. Mc013L has an N-terminal J-domain (43), and a LXXLL motif. The MCV mc013L gene product seems to inhibit ligand dependent transactivation by the vitamin D receptor and the glucocorticoid receptor (M. Buller, personal communication). For this interaction the LXXLL motif and not the J domain is essential. The mc013L protein may function as a blocker of negative growth signals induced by glucocorticoid- and vitamin D receptor-mediated transactivation. This would keep MCV infected epidermal cells in a proliferating state (M. Buller, personal communication). The vitamin D receptor and the glucocorticoid receptor have pleiotropic functions beyond the control of cell growth, e.g. influence on cell differentiation. This is of special interest in the MCV infected epidermis, where dramatic changes in epidermal cell differentiation can be observed (211–213).

MHC Homologues

Many viruses modulate host MHC class I as part of their immune evasion strategy (214). For example adenoviruses 2, 5, and 12 (215–217), human immunodeficiency virus (218), human (HCMV) and murine (MCMV) cytomegalovirus (219,220) down-regulate MHC class I on the surface of infected cells, either by transcription suppression, by enhancing degradation of MHC class I components, or increased internalization of MHC class I through endocytosis. Others like human foamy virus upregulate MHC class I (221).

An alternative approach taken by cytomegalovirus species is the expression of viral MHC homologues (222). In the case of HCMV there is no evidence concerning the biological role of the HCMV MHC class I homologue during viral infection. However, in the case of MCMV disruption of the viral MHC class I homologue produces an attenuated phenotype that is related to increased NK cell mediated cytolysis (220).

The mc080R gene of MCV type 1 and its orthologue in MCV type 2 encodes a major histocompatibility complex (MHC) class I homologue (43). The MHC class I homologue gene of MCV is transcribed early in infection (160). The protein is characterized by a long signal peptide, a C-terminal transmembrane domain, and the fact that a number of amino acid residues thought to be critical for peptide binding by MHC molecules are missing. In a VV expression system the mc080R gene produces two glycosylated polypeptides, a larger unprocessed form with an apparent molecular weight of 47 kDa and a smaller processed form of 42 kDa that represents the MHC class I viral homologue after removal of the signal peptide sequences (223). The protein binds β2-microglobulin, and was not detected on the cell surface. Sequestration of MCV MHC class I homologue in the endoplasmic reticulum and Golgi (223) is consistent with a mechanism that interferes with synthesis and/or transport of cellular MHC class I molecules. Parts of the MCV MHC homologue signal

peptide may be involved in HLA-E mediated inhibition of NK cell activity.

Avipox Cellular Protein Homologues

Recently, five hypothetical fowlpox virus (FPV) genes were found to be present in the virulent precursor, HP1, of the attenuated virus FP9 (224). Two of these genes encode ankyrin repeat proteins that are also common in orthopoxviruses. Three genes encode proteins not found in other viruses. One is a homologue to the yeast Sec17p and mammalian soluble NSF attachment proteins (SNAP). SNAPs are involved in interactions and events leading to vesicle docking and fusion in the exocytic pathway of their respective hosts (225). FPV furthermore encodes a homologue of an orphan human protein, R31240_2, encoded on human chromosome 19p13.2. This hypothetical protein is also homologous to three proteins (YLS2, YMV6, and C07B5.5) from the free-living nematode Caenorhabditis elegans and to a 43 kDa antigen from the parasitic nematode *Trichinella spiralis*. The *Trichinella* protein is associated with invasion of skeletal muscle and initiation of skeletal muscle dedifferentiation (213). The third gene encodes a homologue of the mammalian plasma cell antigen PC-1 and is transcribed early and late in FPV infection (224). Plasma cell differentiation antigen-1 (PC-1) is a type II glycoprotein with exophosphodiesterase activity that has been implicated in insulin- and nucleotide-mediated signalling and cell growth (226). Functional analyses on these FPV hypothetical proteins have so far not been reported.

Possible Mechanisms of Cellular Gene Acquisition by Poxviruses

At this point in the review the obvious question has to be addressed: How can a family of cytoplasmic DNA viruses that do not enter the nucleus and maintain viral replication even in enucleated cells manage to acquire copies of cellular genes? Since the pool of eukaryotic cellular DNA is sequestered in the nucleus, cytoplasmatic DNA viruses lack access to the template and the enzymatic machinery theoretically required for gene transfer based for instance on homologous recombination. However, it remains a fact that despite the unavailability of nuclear mechanisms associated with major genetic variation, e.g. intranuclear homologous recombination, a large number of cellular gene homologues are present in genomes of several members of the poxvirus family. It has to be considered that at least some of them came with the original *protovirus*, meaning that they were acquired as the viruses came into being. As for later acquisitions it has to be assumed that many of these events happened over evolutionary timespans. As a consequence of evolutionary changes in viral base composition and other blurring events, molecular evidence of the mechanism that led to the acquisition in the first place will be hard to come by in most cases. There are a few examples, though that give an idea of the mechanisms involved.

Examples for Plasticity of Poxvirus Genomes

Analysis of restriction fragment length polymorphism (RFLP) reveals that genomes of poxviruses harbour areas of instability (188,188,227–231). The genetically most instable parts are the terminal inverted repeats (TIR) that undergo deletion, reiteration of smaller and reduplication as well as inversion of larger DNA sequence elements by intragenomic recombination during the course of viral replication (232,233). The molluscipox virus MCV harbours multiple regions of low DNA complexity and restriction fragment length variability. Most of them are located in intergenic regions (234–236) but some reside within coding sequences (237). Many aspects of poxviral recombination and its relation to poxviral replication have been studied. Poxvirus genome units are separated by resolution of Holiday structures (238–242). Recombination requires only early gene products, whereas resolution of genome concatemers for replication requires late gene products (243). Recombination, but not replication (see poxvirus encoded IFN inhibitors, this review) is inhibited by IFNγ (244,245). Poxviral recombination is enhanced by the presence of large non-homologies (246,247) and is a highly precise process (248). Integration of endogenous and exogenous plasmid sequences into poxvirus genomes has been described (249). DNA ligase (250) and DNA polymerase are involved in poxvirus recombination but in functions independent from replication (251). Illegitimate recombination occurs in poxviruses and is mediated by the VV topoisomerase (252–257). Exchange of genetic elements between different poxviruses, e.g. intergenomic

recombination, has also been observed and presumably happens during replication of two different poxviruses in the doubly infected host cell. Malignant rabbit virus (MRV) is the result of a natural recombinational event that occurred in wildlife between the two leporipoxviruses myxoma virus and Shope fibroma virus, e.g. in the same poxvirus genus (132,258,259).

Examples for Host Gene Transfer into Large DNA Virus Genomes

The first example of host-derived genes transferred into an eukaryotic virus was the *copia*-like retrotransposon, TED (7510 bp), in the DNA genome of the spontaneous *Autographa californica* nuclear polyhedrosis virus (NPV) few polyhedra (FP) mutant FP-D (260). TED has a retroviral U3-R-U5 structural organisation and the gag-, pol-, and env-like open reading frames of TED encode active enzymes, e.g. protease, reverse transcriptase, and integrase functions, necessary for transposition via an RNA intermediate. TED is able to release infectious retroviral particles from NPV-FP-D infected insect cells (261). Other host DNA insertions into the same region within the genome of NPVs were flanked by short repetitive DNA nucleotide sequence repeats. It was concluded that the NPV ''few polyhedra phenotype'' (FP) was the direct result of TED and other host DNA insertions into the FP-locus of NPVs (262–264). Transpositional mutation of viruses might be a general principle for the horizontal transmission of transposons between species and is made possible and understandable in the case of baculoviruses by their nuclear site of replication. Another family of large DNA viruses rich in viral homologues of cellular genes is the family *Herpesviridae* (for details see Raftery et al., same issue). Certain avian retroviruses and Marek's disease herpesvirus (MDV) are the most common causative agents of avian leukemias and lymphomas. Both viruses are capable of inducing T cell lymphomas in chickens and often coexist in the same animal. MDV vaccines are used to protect the poultry from these diseases. It was found that reticuloendotheliosis virus (REV), a nonacute retrovirus, is able to transfer its proviral DNA into field and vaccine strains of MDV by integrative recombination/insertion (265,266). Coinfection of cultured chicken fibroblasts with avian retroviruses results in stable retroviral insertions into herpesvirus genomes *in vitro*. Insertions are most commonly located in the gD gene and UL/IRL-TR border regions of MDV. This leads to insertional activation or inactivation of herpesvirus genes and results in novel phenotypic properties, e.g. thymic atrophy instead of the neuronal lesions typical for MDV. Full length retroviral integrations into the UL/IRL-TR region of MDV are unstable and frequent deletion events leave solitary retroviral long terminal repeat (LTR) with an occasional concomitant loss of MDV open reading frames at the deletion site (267,268). Solitary LTRs however, are stable in their locations over many passages indicating a past retroviral insertion event in the sense of a ''smoking gun''. Solitary LTRs are found in oncogenic MDV but not in nononcogenic strains (269). Some of the integrated proviruses were infectious when transfected into CEF cells, and therefore could potentially produce infectious REV from a herpesvirus infectious platform (270). Integrated near-full-length sequences from the same REV described above were found in the genome of five field and one vaccine strain (FPV-S) of fowlpox virus (FPV;271). Solitary LTRs were found in the same insertion site in the vaccine strain FPV-M. Free REV in supernatants of FPV-S cultures could not be detected. However, REV particles were produced upon transfection of FPV-S DNA into chicken embryo fibroblasts. Infectious REV can therefore be produced during the infectious cycle of FPV and could even be transmitted via insect vectors, because transmission of FPV by this route seems to be possible (271). Another example for the retrotransposition of host genes into poxviruses may be the VV 16.2-kDa protein (VV strain WR F2L). The F2L gene is transcribed early in infection and its gene product has 31–34% amino acid sequence identity to retroviral protease sequences over a region encompassing 125 amino acid residues (272).

Biochemical Pathways for Host Gene Transfer

In a completely different field of study, the biochemical properties of VV DNA ligase were investigated by Shuman and co-workers (273). VV DNA ligase joins a 3′-OH RNA to 5′-phosphate DNA only slightly less efficiently than 3′-OH DNA to 5′-phosphate DNA and significantly more efficiently than 3′-OH RNA to 5′-phosphate RNA on bridging DNA templates. The RNA-to-DNA strand joining activity of vaccinia DNA ligase may catalyse integration of host cell

RNA into the genome of cytoplasmic poxviruses (273).

Retroviruses, herpes- and poxviruses are naturally occurring pathogens of humans and animals. Coinfection of the same host with both viruses is common. Endogenous retroelements constitute a major part of eukaryotic host genomes. Retroviral enzyme activities could theoretically be present in the cytoplasm of poxvirus infected cells. Retroviral reverse transcriptase can transcribe copies of cytoplasmic mRNA that are then subjected to the significant recombinational activity of the infecting poxvirus enzymatic machinery. This is a feasible pathway for integration of host genes into poxvirus genomes and would explain the lack of introns in poxviral homologues of cellular genes and in fact the genomes of all known large cytoplasmatically replicating virus species. Unorthodox enzyme activities open up possible biochemical pathways in the cytoplasm of infected cells for the acquisition of host genes into poxvirus genomes.

Conclusion

Poxvirus genomes are virtual cDNA libraries of cellular genes (274). From the comparison of cellular genes present in different poxviruses, cowpox virus appears well adapted to its natural hosts with an extensive set of cellular gene homologues, causing little morbidity. In comparison, the loss and inactivation of many orthopoxvirus cellular gene homologues in variola virus suggests a degenerative evolutionary process. In a likely scenario, transmission of the variola ancestor from domestic animals to the human host eventually resulted in a viral phenotype associated with violent infections and high lethality. In contrast, MCV and Orf virus are removed from the rest of the poxvirus family by unique sets of cellular homologue genes. MCV seems genetically especially well adapted to its human host and has perfected longterm survival with little morbidity. Orf virus on the other hand appears as an example of an animal poxvirus that is not well adapted but copes well within accidental human hosts. In contrast, fowlpoxvirus stands completely aside with a unique set of cellular gene homologs derived from its natural avian hosts. With DNA sequence data now available for poxviruses like MCV and Orf virus, it becomes clear that poxviruses benefit from a wide spectrum of host cell gene homologues. Previously, the emphasis has been on gene products that help poxviruses to escape the consequences of the host immune response to infection. Now poxviral homologues of cellular genes that play a role in growth regulation, intracellular signal transduction, free oxygen metabolism, cellular steroid synthesis, and host cell differentiation, all intracellular processes, are moving to the centerstage. The unsolved question how poxviruses acquire new genes inherently involves intracellular events that allow the actual incorporation of host and other foreign genes into poxvirus genomes. In large DNA viruses acquisition of cellular genes with the potential to modify virus virulence and pathogenicity by retrotransposition or new biochemical pathways may be an important mechanism for virus evolution (262,270,271). Genetic events of this type have so far been documented in animal poxviruses and herpesviruses. Similar events in pathogens that cause human disease could have grave consequences (270). Further investigation of the unique pathways underlying these events might help us to understand how poxviruses interact with their hosts on the cellular level and will enable us to further expand the study of poxviruses into the field of cell biology.

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