Analysis of Mutant Pseudorabies Virus Strains

PhD thesis

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LIST OF PUBLICATIONS

List of full papers directly related to the subject of the thesis:

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II. Tombácz D, **Tóth JS**, Boldogkői Z: The Virion Host Shutoff Gene of Pseudorabies Virus Plays a Critical Role in the Transition from the Early to Late Phase of Viral Infection *submitted for publication*

III. Márton G, Tombácz D, **Tóth JS**, Szabó A, Boldogkői Z, Dénes Á, Hornyák Á, Nógrádi A: Ex vivo infection of human embryonic spinal cord neurons prior to transplantation into adult mouse cord. BMC Neurosci 2010, 11:65. **IF: 2,744**

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VI. Prorok J, Kovács PP, Kristóf AA, Nagy N, Tombácz D, **Tóth SJ,** Ördög B, Jost N, Virágh L, Papp GJ, Varró A, Tóth A, Boldogkői Z: Herpesvirus-mediated delivery of a genetically encoded fluorescent Ca2+ sensor to primary adult canine cardiomyocytes. ***J Biomedicine and Biotechnology*** 2009, 361795 **IF: 1,750**

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CONTENTS

LIST OF ABBREVIATIONS

Aa	Amino acid
ADV	Aujeszky's disease virus
ASP	Antisense promoter
AST	Antisense transcript
BICP0	Bovine herpesvirus 1
CAT	Chloramphenicol acetyl transferase
CHX	Cycloheximide
CMV	Cytomegalovirus
CNS	Central nervous system
Ct	Cycle treshold value
Da	Dalton
DMEM	Dulbecco's modified Eagle Medium
E	Early
E	Efficiency of amplification
EDTA	Ethylene diamine tetra-acetic acid
Eg63	Equine herpesvirus's gene 63
ep0	Early protein 0 gene
EWN	Eddinger-Westphal nucleus
FRET	Fluorescence Resonance Energy Transfer
GFP	Green fluorescent protein
HRP	Horseradish peroxidase
HSV-1	Herpes simplex virus 1
i	inhibitory effect
icp4	Infected cell protein 4 gene
IE	Immediate early
IGL	Intergeniculate leaflet
IML	intermediolateral nucleus
L	Late
LAT	Latency-associated transcript

LLT	Long latency transcript
MARCKS	Myristoylated alanine-rich C-kinase substrate
miRNA	Micro RNA
MOI	Multiplicity of infection
OPN	Olivary pretectal nucleus
PAA	Phosphonoacetic acid
PBS	Phosphate buffered saline
pfu	Placque forming unit
PK	Porcine kidney
PRV	Pseudorabies virus
PRV-Ba	Strain Bartha of PRV
PRV-Ka	Strain Kaplan of PRV
PVN	paraventricular nucleus
R	Relative expression ratio
RING	Really interesting New Gene
rr	Ribonucleotid reductase gene
RT^2-PCR	Reverse transcription Real-Time PCR
SCG	superior cervical ganglion
SCN	Suprachiasmatic nucleus
SDS	Sodium dodecyl sulfate
SHV-1	Suid herpesvirus 1
TFP	Teal fluorescent protein
Tm	Melting temperature
ul	Unique long
us	Unique short
UT	Untreated
vhs	Virion host shut off gene
VP16	Virion protein 16
VZV	Varicella zoster virus

INTRODUCTION

The Virus

Pseudorabies virus (in short: PRV; also known as Aujeszky's disease virus (ADV) or suid herpesvirus (SHV-1)) is a well-known pig pathogen responsible for Aujeszky's disease. The earliest reports on the disease were published in 1813 in the United States. In 1902 a Hungarian veterinarian, Aladár Aujeszky, isolated PRV from dog, ox, and cat and showed that it caused the same disease in swine and rabbits [1]. The name *pseudorabies* came from the symptoms similar to rabies that it caused in rabbits. PRV infects a wide variety of domestic and wild animals, however, humans and higher primates are resistant to PRV infection [2].

PRV belongs to the subfamily of *Alphaherpesvirinae,* which is also called as *neurotropic herpesviruses. Alphaherpesviruses,* such as herpes simplex virus type 1 and 2 (HSV-1,-2), varicella- zoster virus (VZV), and PRV, have a similar gene arrangement and share considerable amino acid sequence homologies in their gene products [3]. Hence, PRV is a relevant model to study the biology of *Alphaherpesviruses*. Furthermore, PRV is easy to propagate in cells of several mammalian species including rodents and it is not harmful to laboratory workers. Because of its neurotropic nature, PRV has also been used as a tool to trace circuits in the mammalian nervous system [4] and it has also reported to serve as a suitable tool for gene delivery to various cell lines [5].

Life cycle

Pseudorabies viruses can undergo a lytic (productive) or a latent infection. During productive infection of HSV-1 the transcription of the immediate early (IE) genes is activated by VP16, which is the HSV tegument's major component [6]. TAATGARAT (R means a purine nucleotide) is the target DNA sequence of VP16. This sequence is present in at least one copy in all HSV IE promoters. TAATGARAT is the binding site for the cellular factor Oct-1, which was initially characterized by the ability to bind the octamer sequence ATGCAAAT [7]. VP16 and other cellular proteins bind to the Oct-1/TAATGARAT complex, by this means bringing the VP16's C-terminal domain into proximity with the preinitiation complex. Pseudorabies virus early protein 0 (EP0) may play the same role as VP16, that is, EP0 is supposed to act in combination with IE180 protein, the major regulatory protein of PRV [8]. During a latent infection the viral

genome is transcriptionally silent, with the exception of latency-associated transcripts (LATs), which accumulate to high levels [9, 10, 11, 12 and 13]. LAT is encoded in the complementary DNA strand *ep0* gene, suggesting that LAT inhibits *ep0* expression by a yet unrevealed antisense mechanism. The LAT promoter (LAP) shows neuronal specificity and is also associated with the prevention of apoptosis of neuronal cells [14]. Reactivation from latency can be achieved by external stimuli (e.g. stress or immunosuppression). The ability of PRV to reactivate from latency results in persistent disease and virus transmission [15].

Viral structure

PRV genome is composed of a 142-kb linear double stranded DNA molecule, which contains two components, a unique long (L) and a unique short (S) region. With regard to the orientation of L and S components relative to each other, the viral DNA consists of two equimolar isomers [16]. PRV contains 70 protein coding genes. The genome of PRV is enclosed within an icosahedral capsid, which is embedded in a protein matrix (called tegument) and the latter is surrounded by the envelope, a lipid membrane containing numerous viral glycoproteins [2].

Gene expression

Gene expression of PRV is clustered into three main kinetic classes: immediate-early (IE), early (E) and late (L) phases during a productive infection. The expression is regulated in a cascade like-fashion. IE genes are synthesized directly following infection and they switch on the transcription of the next kinetic class of genes, the early genes. These genes are generally involved in DNA replication and nucleotide metabolism. The late genes are synthesized after viral replication and mainly encode the structural proteins of the virions, including capsid proteins and viral glycoproteins. The transcription of late genes is regulated by *ie180*, the only IE gene of PRV [17] and some E gene products [2]. The product of *ie180* gene has been shown to be homologous to that of ICP4 protein of HSV-1. IE180 functions as a strong transactivator on different promoters and as a repressor on its own transcription [18]. Nevertheless five IE genes have been described in HSV-1, four of them participate in controlling gene expression (*icp4, icp0, icp27* and *icp22*), while one (*icp47*) interferes with antigen presentation [19]. In PRV *ep0* (homolog of HSV *icp0*) and *ul54* genes (homolog of HSV *icp27* gene) are expressed with early

kinetic, while PRV lacks the *icp47* gene. There is a debate as to whether PRV *us1* gene (homolog of HSV *icp22*) is expressed with an IE or an E kinetics [20].

Earlier, the kinetic classes of genes were characterized by Northern and/or Western blot techniques or later with microarray analysis. Nevertheless, these techniques have numerous disadvantages. For example, Northern blotting is time-consuming and labor-intensive, allows only semi-quantitative determination of the mRNA level. Over the past decade, the DNA chip techniques have revolutionized practically all disciplines of molecular biology, including large genome virus research. One of the biggest disadvantages of DNA chip technology is associated with the uncertain quality control: it is impossible to assess the identity of DNA immobilized on any microarray. Also during microarray experiments in the laboratory, sequence homologies between clones representing different closely related members of the same gene family may result in a failure to specifically detect individual genes and instead may hybridize to spot(s) designed to detect transcript from a different gene. This phenomenon is known as cross hybridization. Further, fluorescence technology, which is the most commonly used detection method for array readouts is reproducible, but is limited in sensitivity and there are many artifacts associated with image and data analysis. Despite its disadvantages microarray has been recently applied technique to investigate herpesvirus gene expression. Real-time RT-PCR is an alternative to microarray techniques for the analysis of transcription from multiple genes. RT-PCR (reverse transcription-polymerase chain reaction) is the most sensitive technique for mRNA detection and quantitation currently available. Compared to the two other commonly used techniques for quantifying mRNA levels, Northern blot analysis and microarray analysis, RT-PCR can be used to quantify mRNA levels from much smaller samples. In fact, this technique is sensitive enough to enable quantitation of RNA from a single cell. To date, Real-time RT-PCR has not been frequently utilized in herpesvirus research for global gene expression analysis [21, 22 and 23].

PRV genes

The ie180 gene

PRV expresses a single immediate-early (IE) protein from two copies of the IE gene that can be found in both inverted repeats of the viral genome. The *ie180* gene encodes a 180-kDa protein (*ie180*) consisting of 1460 amino acid residues that is essential for initiation of the early and late gene expression and represses the synthesis of its own RNA [18]. The *Ie180* shows a high degree of homology with the immediate early protein of other alphaherpesvirus, i.e., *icp4* of herpes simplex virus type 1 (HSV-1). Without expression of the IE gene, the switch from the IE phase to the early and late phases of PRV gene expression does not occur, and therefore the viral replication is aborted [24]. The promoter region of the IE gene has been well characterized [25]. The IE promoter contains nine imperfect repeats; each consists of five to six different consensus elements for the binding of transcription factors [26]. The IE promoter contains numerous conserved regulatory elements, for example the transcription factor Sp1 binding sites, the TAATGARATTC motifs, the CCAAT boxes, the GCGGAA motifs and the repeated sequence which possibly plays a role in autoregulation of IE gene's transcription [27]. This sequence is recognized by IE180 protein. The PRV IE promoter is activated directly after the viral infection. Because this promoter regulates its own protein's expression, it plays an important role in the activity of this promoter and probably determines the viral tissue specificity and pathogenicity [28]. The PRV IE promoter drives a pan-specific expression in the transgenic mice, and the activity is constantly high in the neuronal tissues. In vitro analysis of mutated IE180 mutants has identified three functionally essential domains: the acidic transcriptional active domain, the DNA-binding domain and the nuclear localization signal. All these domains are required for the transactivation, and the latter two are also essential for autoregulation of IE180 gene itself [29].

The early protein 0 (ep0)

The early protein 0 is a very important regulatory proteins of PRV, which is expressed at the early stage of virus infection[30]. Ep0 gene is located in the unique long region of the PRV genome. A polyadenylated 1.7-kb transcript encodes the 409-aa PRV EP0 nuclear phosphoprotein. The amino acid sequence of EP0, especially the amino-terminal C3HC4

RING (**R**eally **I**nteresting **N**ew **G**ene) finger domain, shares homologies with HSV-1 **I**nfected-**C**ell **P**olypeptide 0 (ICP0) and VZV open reading frame 61 (ORF61) protein, BICP0 of bovine herpesvirus 1 and the equine herpesvirus's gene 63 (Eg63) [31]. It is thought that EP0 may play an important role in DNA-binding, RNA-binding, or protein-protein interactions [32]. Previous studies exposed that EP0, ICP0, and ORF61 protein are able to activate the expression of all classes of alphaherpes viral genes such as ICP4, thymidine kinase (TK), gB, gC, gD and gG, as well as cellular genes, such as (1) elongation factor 1α, (2) ubiquitin-specific protease or (3) PML [33, 34, 35, 36 and 37]. In our days important regions have been precisely located within the sequence of ICP0, namely the nuclear localization signal, the USP7 binding domain (594 to 633 amino acids) [38], and the multimerization C-terminal sequences (633 to 711 amino acids) [39 and 40]. Hence, to define the extent of sequence similarity between ICP0 and its homologues BICP0, Eg63, ORF61, and EP0, the amino acid sequence homologies were analyzed by Parkinson and Everett. It has been shown that no other obvious sequence similarities are present in PRV EP0 and its alphaherpesvirus homologues, except of the RING finger domain, and none of the ICP0-related proteins showed sequence similarities to the well-defined USP7 binding region (Table 1) [41].

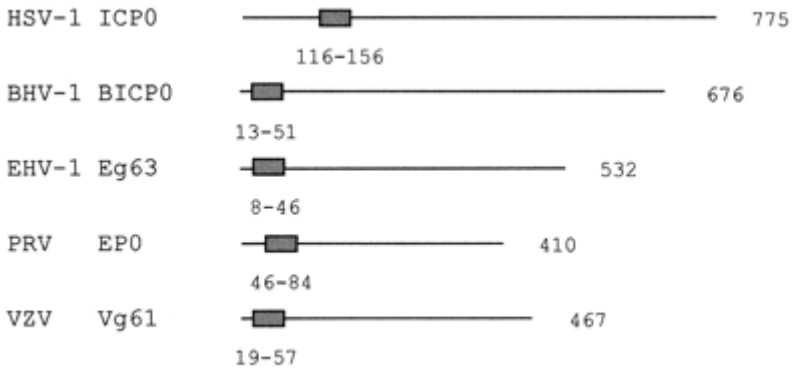


Table 1. Localization of RING-finger domain in alphaherpesviruses [42]

The expression of *ep0 in vivo* initiates gene expression from PRV promoters, such as *ie180*, *ul23*, and us*4*, and other viral promoters in HSV and VZV. Whether EP0 acts directly or indirectly in modulation of transcription is not yet established. Ho and co-workers publication describes [43], that PRV EP0 activates the TATA-containing promoters through facilitating the transcription initiation, suggesting that PRV EP0 has a

preference for specific core promoter. In addition, this protein (and its homologues in HSV) is able to interact with the components of initiation complex [44, 45 and 46]. Further, the expression of *ep0* gene plays a key role in enhancement of reactivation from quiescence, by disruption of ND10 structures, induction of proteasome-dependent degradation of cellular proteins, and interaction with cyclin D3 [2]. It is still unclear whether EP0 associates with the envelope, tegument or capsid protein. Ono and co-worker showed that EP0 is a virion component, and since EP0 is an activator of the IE gene, it is presumably located in the tegument, such as the strong transactivator, VP16, of the HSV-1 IE genes [8].

The virion host shut off gene (vhs)

Virion host shutoff (VHS) protein is the product of *ul41* gene that induces rapid shutoff of host cell and viral protein synthesis. The *ul41* is expressed as a late gene during infection, then is packaged into virions and in newly infected cells, where it degrades the preexisting and newly transcribed mRNAs [47]. The *ul41* expression results in two forms of the VHS protein, the 59.5 kDa phosphoprotein and the 58 kDa polypeptide [48]. These proteins are essential in the early stage of shutoff phenomenon. The delayed shutoff happens due to the expression of *ul54* gene, which role is the shutoff of host protein synthesis. The VHS protein does not differentiate between cellular and viral mRNAs, but it shows a strong preference for mRNAs, as opposed to rRNA and tRNA and this indicates that *vhs* acts as an mRNA-specific RNase [49]. HSV *vhs* and its orthologues in the other alphaherpesviruses show significant similarity in their amino acid sequence to a family of cellular nucleases that participate in DNA replication and repair, such as FEN-1 (non-viral proteins), which is an endo/exonuclease that removes the RNA primers from Okazaki fragments during DNA replication in eukaryotes [50].

Ribonucleotide reductase (rr) gene

Ribonucleotide reductase (RR) is an essential component of the *de novo* pathway for deoxyribonucleotide synthesis and is required to elicit acute infection in pigs and mice. The RR subunits are encoded by the genes *ul39* (the large subunit, or RR1) and *ul40* (the

small subunit, or RR2). Deletion of *rr* gene made PRV avirulent, however *rr* strains retained their ability to form viable viral particles in cultured cells [51 and 52].

Glycoprotein E and glycoprotein I (gE, gI)

The tegument of extracellular virions is localized between the capsid and the envelope of the virus. The tegument of *Alphaherpesvirinae* consists of at least 20 viral encoded proteins [53 and 54]. Loret and colleagues suggested that the actual number of viral protein is 23 which have numerous functions during viral replication [55]. Such roles are (1) the capsid transport during entry and egress, (2) targeting the capsid to the nucleus, (3) regulation of viral-gene expression, (4) DNA replication, (5) immune modulation, (6) cytoskeletal assembly and (7) nuclear egress of capsid. Originally HSV-1 virion proteins were named as viral protein (VP) 1 to VP24, based on their migration in a protein gel [56]. HSV-1 tegument proteins are also called as infected cell polypeptide (ICP). It was known that a number of VPs represented differential posttranslational modification of the same tegument protein and were encoded by single gene. For example, VP11 and VP12 are both encoded by the gene *ul46* and is referred to as VP11/12 or p(protein) UL46. The prefix "p" was adopted to distinguish between gene and protein for both HSV-1 and PRV [57]. The exceptions to this nomenclature (which was generated at the 18th International Herpesvirus Workshop in 1993) are the tegument proteins ICP0, ICP4 and ICP34.5, whose genes are localized at the repeat regions of the virus. In HSV-1, most of the tegument proteins, pUL46 (VP11/12), pUL47 (VP13/14), pUL48 (VP16), pUL49 (VP22) are present at 1000–2000 copies while the rest are present at less than 1000 copies. As reported by del Rio et al. for PRV, deletion of nonessential tegument proteins is compensable by increased incorporation of other tegument proteins such as pUL46, pUL48 and pUL49 or cellular proteins such as actin [58]. PRV has at least three nonessential membrane proteins which are important for directional spread of virus from neuron to neuron. The type I transmembrane glycoproteins E (gE protein, encoded by *us8* gene) and I (gI protein, encoded by *us7* gene), and the type II membrane protein US9 are necessary for spread from presynaptic retinal ganglion cells to postsynaptic neurons. In pigs, *gE* and *gI* are essential for PRV to passage from presynaptic olfactory neurons to

postsynaptic neurons. All viruses deleted for these genes spread in a reverse order, from postsynaptic to presynaptic neurons.

The gE and gI form a hetero-oligomer that promotes the maturation and intracellular transport of these proteins to the plasma membrane of cells. It is still unknown that US9 forms a complex with gE or gI, or it may act at another step to subserve transneuronal spread of PRV. Infection of non-neuronal (for example: Madin-Darby bovine kidney) cells by *gE* and *gI* null mutants, results in formation of small plaques suggesting that *gE* and *gI* may be essential for efficient cell-to-cell spread of the virus. The analysis of truncated gE protein showed that the gE protein's N-terminal extracellular domain is adequate for gE-gI complex formation, and for anterograde spread of PRV, while the C-terminal cytoplasmic domain of gE is necessary for CNS infection through anterograde and retrograde transport of virus [59].

Little is known about the kinetics of PRV US7 expression, though its gene product can be detected late (6 hours) after infection at high MOI in PK15 cells. Expression kinetic is not well characterized in HSV-1 either, where US7 is thought to be a leaky-late (γ1) gene [60].

PRV US8 transcripts are absent at 2 h pi but appearing at 3 to 5 h pi following infection at very high MOI (100 pfu/cells) [60]. US8 transcript was characterized as an early gene, while its HSV-homolog was described either as an early or as a late gene; so there is no consensus on this issue [61].

Genomic sequences

Antisense Promoter (ASP)

The putative antisense promoter can be found at the PRV's inverted repeat region; therefore, it is represented in two copies. The presumed role of ASP is to control the expression of an antisense transcript called AST (antisense transcript), but the exact function of this transcript is not known yet. Boldogkői et al published in 2002, that a single point mutation (within the TATAA box of the promoter) in this region leads to substantial reduction of virulence in PRV [51].

Neut region

The PRV BamHI8' fragment can be found between the *ie180* and *ep0* genes and contains numerous unique restriction sites. Mutations within this region are neutral with respect of the virulence of PRV.

Antisense RNAs

The traditional view of transcriptional regulation has dramatically changed in the past few years, because it has become evident that the majority of complex organism's genome is transcribed to produce large numbers of non-coding (nc) RNAs, which are antisense and intergenic or overlapping with protein-coding genes. Such ncRNAs function by base-pairing with complementary sequences in other RNAs or DNAs. These ncRNAs can be categorized into two major groups, *cis* and *trans*. Example for the former contain sequences in UTRs that bind regulatory proteins or to be targets of RNA editing to control the translatability, stability or localization of mRNAs. The regulation by cis-(overlapping) RNAs was described by Spiegelman et al. in 1972 [62], who worked on the gene regulation of bacteriophage λ, and found that these transcripts are common in viruses, bacteria and eukaryotes. The possible function of these ncRNAs is the formation of dsRNAs, which are cleaved into small interfering RNAs (siRNAs) that can directly modulate the gene expression. Examples of the trans-acting RNAs are the "riboswitches" that can regulate metabolic pathways by binding e.g. vitamins or amino acids to control mRNA translation or stability [63]. In the 1980s it was discovered in *Escherichia coli* that small RNA molecules (about 100 nucleotides in length) bind to a complementary sequence in mRNA and inhibit translation [64]. Currently, about 60 of regulatory trans-acting antisense RNAs are described in *E. coli*. Translation regulation trough antisense RNAs in eukaryotes was first demonstrated in 1993 [65 and 66], when genes regulating the development of *Coenorhabditis elegans* were studied [67]. The idea of this regulatory mechanism became more accepted, when a second example of small regulatory RNA was discovered in *C. elegans*, because in that case similar sequences were found in other species. Nevertheless, the situation changed radically when a large number of microRNAs (miRNAs), were revealed in 2001 [68, 69 and 70]. "Infrastructural" ncRNAs, like rRNAs, tRNAs, spliceosomal uRNAs and small nuclear RNAs (snoRNAs)

are required for both translation and splicing. But recent findings suggest that some of these RNAs may also be involved in regulatory processes. For example, U1 RNA (besides its role in splicing) interacts with cyclin H [71], posing the possibility that this ncRNA is involved in cell cycle regulation. Nowadays, there is growing evidence that these ncRNAs are functional; they also may have a role in the regulation in epigenetic processes [72].

In PRV, LLT (long latency transcript, also called as antisense transcript; AST) and LAT (latency-associated transcript) overlap the *ie180* and *ep0* genes (a homologue of *icp4* and *icp0* in HSV), respectively, have been described to play important roles in the establishment of latency [2 and 73]. It has not yet been unequivocally clarified whether the expression of antisense transcript produced by the complementary DNA strand of the *ie180* gene is solely controlled by the LAP (LAT promoter) producing LLT (AST) or also by a putative promoter (antisense promoter, ASP) localized on the inverted repeat of the PRV genome, producing a shorter transcript [16]. In this thesis, I will use the term 'antisense transcript' (AST) for the RNA molecule transcribed from the complementary DNA strand of the *ie180* gene.

Herpesviruses in neurobiology

The central nervous system (CNS) is a complex network of synaptically linked neurons. To understand the function of this complex network, it is needed to distinguish between the functionally related and the non-related neurons; hence, various labeling techniques were developed in the past decade [74]. The non-viral labeling methods are almost based on microtubular axonal transport mechanism [75] and are useful for retrograde (opposite to the direction of impulse transmission; determination of the somata's location of labeled afferent nerve fibers) tracing. The first such conventional tracer was the horseradish peroxidase (HRP), a plant enzyme, which could be taken up by the axon and moved to the cell body [74]. Nowadays HRP, TrueBlue and FastBlue are commonly used tracers, nonetheless numerous disadvantages of these dyes are known, like they are unusable for multiple labeling and cannot cross synapses. The natural spreading mechanism of neurotropic herpesviruses, like PRV allow them to be suitable candidates for gene delivery into the nervous system. The replicating virus has the ability to spread through

the synaptically linked neurons, while attenuated strains (e.g. the Bartha strain), are used in neuroanatomical tracing experiments, because it spreads in a retrograde manner and are able to deliver large or mutated foreign DNA fragments. [76]. Recently, PRV become the most important viral tracer in neuroscience, because by genetic modifications (namely with deletion of *gE, gI, us9* genes) all the requirements can be fulfilled, that are essential for an efficient neuronal circuit tracer. These requirements are as follows: low virulence, specific and retrograde spread. In several studies was pseudorabies virus used to map neural circuits [77, 78 and 79]. Transsynaptic pseudorabies viruses expressing various cytosolic or membrane-bound fluorescent proteins are effective for labeling neurons in the same functional circuit within a complex mesh of local neural circuits in vivo or in vitro. Furthermore, the activity of neurons can also be monitored by using genetically modified PRV expressing genetically encoded fluorescent Ca^{2+} sensors. Furthermore, PRVs encoding two differently colored fluorescent proteins can be utilized for revealing the synaptic orders of the examined neural pathway[4].

Herpesviruses in cardiovascular research

As far as we know, no other research group used genetically modified herpesviruses for delivery and expression of foreign DNA sequences into cardiomyocytes.

The aims of the studies

Gene expression analysis

Global gene expression of PRV under different experimental conditions. We examined

1., global expression of PRV genes in CHX and PAA treated and non-treated cells

2., the dependence of expression properties of 37 PRV genes on the multiplicity of infection

3., the effect of *vhs*- knock-out virus strain on the expression of other PRV genes

PRV in neurobiology

Development of transgenic viruses that are expressing an activity sensor and multiple colored fluorescent proteins. With the help of these viruses we can differentiate between

pre- and postsynaptic neurons, measure the activity of these connected neurons and determine the time-window for functional analysis.

PRV as a tool in cardiovascular research

Our aim was to use fluorescent protein encoding PRVs as gene delivery vectors for cardiomyocytes. An additional aim was to monitor the effect of recombinant viruses on cell viability.

Remark: In this thesis, I will focus more on PRV gene expression studies, which were my main projects, than on the use of PRV as a tool in biological disciplines, in which I participated as a co-worker.

MATHERIALS AND METHODS

Cell lines

Porcine kidney 15 (PK-15) cells

Monolayer cultures of immortalized porcine kidney 15 (PK-15) cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich) supplemented with 5% fetal bovine serum (Gibco) and 80 μg gentamicin (Invitrogen^{TM}) per ml at 37°C with 5% CO_2.

Cell viability analysis of canine cardimyocytes

Freshly isolated myocytes were centrifuged five times in sterile 10% PBS. The supernatant was replaced first by 500 μM then by 1mM Ca^{2+} containing PBS solution. Precipitated cells were resuspended in culture medium and plated on laminin coated (1 μg/cm2) sterile cover glass. Cells were left 4 hours at 37◦C under sterile conditions in an incubator ventilated with 5% CO2 and 95% air to attach to the 6-well-plate and after this time period non attached cells were washed out, and the rest was infected with various titers of recombinant pseudorabies viruses. Following the first medium (Culture medium consisted of serum-free medium 199 (M199) supplemented with 25mM NaHCO3, 5mM ceratine, 2mM L-carnitine, 5mM taurine, 100 units/mL insulin (CCTI supplemented medium) and 50 μg/mL gentamycin) change, subsequent medium changes were carried out every day. Morphological changes of cells were observed by light microscope on a

daily basis parallel with physiological measurements. Troponeon-positive cells were examined by fluorescence microscopy from one to three days following isolation at standard titer of viruses. Infection efficacy was determined separately for infected cells by manual cell counting using a fluorescent microscope (Olympus IX-71) [5].

Viruses and infection

Virus strains

Strain Kaplan of pseudorabies virus (PRV-Ka) was used for gene expression analysis and for developing some of recombinant virus strains (Ka-gEI-Orange, Ka-VHSlac-gEIΔDupCFP, PRV-rrep0lacgfp, Ti-PRV08) [4 and 76]. PRV Bartha (PRV-Ba) strain was also employed for producing recombinant PRVs (Ba-DupmemTFP, Ba-DupGreen, Ba-NeutRed [80]. For the gene expression study Ka-vhsΔ strain was applied. Viruses were maintained in cultured of immortalized porcine kidney cells.

Viral stock preparation and infection

The virus stock used for the gene expression experiments was prepared as follows. Rapidly-growing semi-confluent PK-15 epithelial cells were infected at an MOI of 0.1 pfu/cell and were incubated until a complete cytopathic effect was observed. The cell debris was removed by low-speed centrifugation (10,000 g for 20 min). The supernatant was concentrated and further purified by ultracentrifugation through a 30% sugar cushion at 24,000 rpm for 1 h, using a Sorvall AH-628 rotor. The sedimented virus was resuspended in sodium Tris-EDTA buffer. After the addition of proteinase-K (l00 μg/ml final concentration) and sodium dodecyl sulfate (SDS; 0.5% final concentration), the lysate was incubated at 37°C for 1 h, which was followed by phenol-chloroform extraction and dialysis. The number of cells in a culture flask (Corning, 150 cm^2) was $5x10^6$. In high-MOI and in low-MOI experiments, $5x10^7$ and $5x10^5$ pfu viral particles, respectively, were applied for the infections. Thus, in the high-MOI experiment, practically all the cells were infected, while in the low-MOI experiment, approximately $5x10^5$ cells ((10% of the cells in a culture flask) were infected by the virus) were infected at a MOI of 0.1 or 10 for the gene expression analysis. The two experiments were run simultaneously. We ran four independent sets of measurements for each time point in both low and high-MOI studies, but occasionally we had to remove data because of low

amplification efficiencies or the amplification of non-specific products in the reaction. Thus, in some genes, instead of four, we only used three independent data. Infected cells were incubated for 1 h, followed by removal of the virus suspension and washing with phosphate-buffered saline (PBS). After the addition of new medium to the cells, they were incubated for 0, 1, 2, 4 or 6 h. For the analysis of antisense transcripts, cells were also treated with 100 μg/ml cycloheximide (CHX), a protein synthesis inhibitor, or with 400 μg/ml phosphonoacetic acid (PAA), inhibitor of DNA replication 1h prior to low-MOI virus infection.

For the examination of knock-out virus stains, cells were infected with high (10 pfu/cell) multiplicity of infection of wild-type (Ka-strain), vhs-mutant viruses and were incubated for 1, 2, 4, 6, 8, 12, 18 or 24h. In our studies, mock-infected cells were used as controls, which were otherwise treated in the same way as the infected cells. The virus stock for PRV-based delivery system was made by infecting PK-15 cells with 1 pfu/cell and then harvesting them for 24h. After this step cells were freezing and thawing three times then cells were centrifuged and the pellet was discard. The supernatant was stored at -80 C° in 400 μL aliquots until further use.

RNA preparation

RNA was extracted by using the NucleoSpin RNA II Kit (Macherey-Nagel GmbH and Co. KG), as described previously [21]. Briefly, after the cells had been collected by centrifugation and lysed by buffer containing chaotropic ions, the nucleic acids were docked to a silica column. The DNA was removed with RNase-free DNase solution (supplied with the NucleoSpin RNA II Kit). Finally, the RNAs were eluted from the column in RNase-free water (supplied with the kit). To eliminate the residual DNA contamination, all RNA samples were treated by an additional digestion with Turbo DNase (Ambion Inc.). The concentrations of the RNA samples were measured by spectrophotometric analysis with a BioPhotometer Plus instrument (Eppendorf). RNA samples were stored at -80 °C until further use.

Quantitative real-time RT-PCR

A two-step quantitative real-time RT-PCR was carried out for the transcriptional analysis.

Reverse transcription (RT)

Total RNA isolated from infected cell cultures and was reverse-transcribed into cDNA for PCR analysis. RTs were performed in 5 µl of solution containing 0.07 µg of total RNA, 2 pmol of the gene specific primer, 0.25 µl of dNTP mix (10 µM final concentration), 1 µl of 5× First-Strand Buffer, 0.25 µl (50 units/µl) of SuperScript III Reverse Transcriptase (Invitrogen) and 1 U of RNasin (Applied Biosystems Inc.) and the mixture was incubated at 55°C for 60 min. The reaction was stopped by raising the temperature to 70°C for 15 min. No-RT control reactions (RT reactions without Superscript III enzyme) were run to test the potential viral DNA contamination by conventional PCR. RNA samples with no detectable DNA contamination were used for quantitative RT-PCR reactions.

Real-time PCR

First-strand cDNAs were diluted 10-fold with DEPC-treated water (Ambion Inc.) and after that subjected to real-time PCR analysis. Real-time quantitative PCR experiments were performed with a Rotor-Gene 6000 cycler (Corbett Life Science). All reactions were carried out in 20-µl reaction mixtures containing 7 µl of cDNAs, 10 µl of ABsolute QPCR SYBR Green Mix (Thermo Fisher Scientific), 1.5 µl of forward and 1.5 µl of reverse primers (10 µM each). The running conditions were as follows: (1) 15 min at 95°C, followed by 30 cycles of 94°C for 25 sec (denaturation), 60°C for 25 s (annealing), and 72°C for 6 s (extension). The absence of nonspecific products or primer dimers was indicated by observation of a single melting peak in melting curve analysis. An additional extension and detection step was applied for those primers that produced primer dimers: for 2 s at a temperature just below the Tm (melting point) of the specific product and substantially above the Tm of the primer dimers. With this technique we could eliminate nonspecific fluorescent signals produced by primer dimers. Following the PCR reaction, melting curve analysis was performed to control amplification specificity (specificity was defined as the production of a single peak at the predicted temperature and the absence of primer dimers) by measuring the fluorescence intensity across the temperature interval

from 55°C to 95°C. The 28S ribosomal (r)RNA was used as the loading control (reference gene) and was amplified in each run. H_2O was included as a no-template control, and cDNA derived from the reverse-transcribed RNAs of non-infected cells was used as a negative mock-infected control. We applied SYBR Green-based real-time PCR because of the lower costs and simpler protocol than for TaqMan probe-based methods for instance [21]. It has recently been demonstrated that the SYBR-based method of detection is as sensitive and specific, and has a similar dynamic range to that of the TaqMan-based technique [81].

Data analysis

The following formula was used for calculation of the relative expression ratio (R):

$$R=\frac{\overline{(E_{sample6h})^{Ct_{sample6h}}}}{\overline{(E_{sample})^{Ct_{sample}}}} : \frac{\overline{(E_{ref6h})^{Ct_{ref6h}}}}{\overline{(E_{ref})^{Ct_{ref}}}}$$

where R is the relative expression (quantification) ratio; E is the efficiency of amplification; Ct is the cycle threshold value; sample refers to any particular gene at a given time point; and ref is the 28S rRNA, which was used as a reference gene and was amplified in each run. Instead of individual values, we applied the average maximal value of E^{Ct} for each gene as the control. The relative copy numbers of mRNAs were calculated by normalizing cDNAs to 28S rRNA using the Comparative Quantitation module of the Rotor-Gene 6000 software (Version 1.7.28, Corbett Research), which automatically calculates the real-time PCR efficiency sample-by-sample. Thresholds were set automatically by the software.

Data were analyzed by the Microsoft Excel program, the average and the standard deviance functions of the software were used. The inhibitory effect of CHX or PAA was calculated via the ratio of the drug-treated and untreated (UT) R values at 2, 4 and 6 h pi for CHX: $R_{i\text{-}CHX} = R_{CHX}/R_{UT}$, or 4 and 6 h pi for PAA: $R_{i\text{-}PAA} = R_{PAA}/R_{UT}$. Thus, a low value indicates a high inhibitory effect and vice versa. To measure the net change in R between two consecutive time points, R_Δ was calculated via the following formula: $R_\Delta = R_{(t+1)}\text{-}R_t$. The rate of change was calculated as follows: $R_a = R_{(t+1)}/R_t$. The effect of VHS protein on the gene expression was calculated by using the R_r values, a ratio between the

R values of *vhs*-knockout and *wt* PRVs ($R_r = R_{\Delta vhs} / R_{wt}$), where $R_{\Delta vhs}$ and R_{wt} represent the R value of a particular gene at a given time point in *vhs*-knockout and wild-type genetic background, respectively. A high R_r value indicates an excessive inhibitory effect of the VHS protein on the transcript level of a particular gene in the wild-type virus. Furthermore, we have calculated the average R_r for the E, E/L and L genes for every time points: $\overline{R}_r = \overline{R}_{\Delta vhs} / \overline{R}_{wt}$. Pearson's correlation was used for the analysis of the relationship between low- and high-MOI and wild-type and mutant virus infections. Pearson's correlation coefficient (r) was calculated as follows.

$$r = \frac{\sum_{i=1}^{n}(X_i - \overline{X})(Y_i - \overline{Y})}{(n-1)S_x S_y}$$

The correlation measures the linear relationship between two variables, X and Y. Pearson's coefficient (r) is a number ranging from -1 to +1 that measures the degree of association between X and Y. If X and Y are independent, Pearson's correlation coefficient is 0. A positive r value for the correlation implies a positive association (large values of X tend to be associated with large values of Y, and small values of X tend to be associated with small values of Y). A negative value for the correlation means an inverse association (large values of X tend to be associated with small values of Y, and vice versa). For gene expression analysis the above mentioned formulas were used [21].

Construction of recombinant viruses

Targeting plasmid vectors were used to deliver reporter genes and/or mutations to the PRV genome. Homologous recombination between the targeting vector and the virus was used to deliver foreign sequences of a mutation to the PRV genome. After isolation of targeted virus DNA and sub-cloning it to a plasmid vector, different colored fluorescent protein coding genes and activity sensor markers were inserted into the cloned viral sequence. After this the viral DNA was transfected along with the targeting plasmid to PK-15 cells, where the homologous recombination took place. The recombinants were then isolated on the basis of the fluorescence of the infected cells.

Flanking sequences

PRV DNA segments of interest were subcloned into members of a palindrome-containing positive-selection vector family (pRL479, pRL525, pRL494) [82]. Subsequently, viral DNA sequences were cut with one or two unique restriction endonucleases, followed by Klenow filling-in of 5'-overhangs and insertion of either an *Eco*RI or a *Hind*III linker, which served as a cloning site for the incorporation of reporter gene expression cassettes [4].

Reporter genes

For creating membrane targeted FPs, reporter genes (e.g. Orange, memTFP or CFP) were fused with MARCKS2 (myristoylated alanine-rich C-kinase substrate). Each expression cassette was modified to contain either *Eco*RI or *Hind*III restriction endonuclease sites at both ends for subcloning into flanking viral sequences [4].

gE-gI (glycoprotein E and I gene)-mutant

The *Bam*HI-7 PRV DNA fragment was isolated and subcloned into the pRL525 vector to give p525-B7. The 1855-bp *Stu*I – *Age*I DNA fragment of p525-B7 was replaced by an *Eco*RI linker generating pgEgI-RI. Removal of the *Stu*I – *Age*I DNA fragment resulted in the inactivation of both gE and gI genes of the virus.

vhs (virion host shut-off gene)-mutant

The 2526-bp XhoI DNA fragment containing the entire VHS gene was subcloned into the SalI site of pRL494 resulting in p494-Xh. The unique NruI site of this DNA segment was converted to an EcoRI site (generating pVHS-RI) by linker insertion, which resulted in a frameshift mutation in the VHS gene. LacZ gene was inserted in the unique EcoRI site and the former was controlled by a CMV promoter. This construct was transfected along with wild-type (Ka) PRV in cultured cells, where homolog recombination occurred.

ep0 (early protein 0)-mutant

The *Kpn*I-F fragment of the viral genome was cloned into the *Kpn*I site of pRL425, then the *Bam*HI-14 fragment of 1 390 bp containing almost the entire *EP0* gene, was deleted from the plasmid by *Bam*HI digestion followed by ligation of the DNA ends. This

plasmid was treated with *Bam*HI and Klenow enzyme, and then *Hin*dIII linkers were attached to DNA, resulting in pΔEP0. The *Hin*dIII fragment of the expression cassette was engineered into the *Hin*dIII site of pΔEP0, resulting in the transfer plasmid (pΔEP0lac). Plasmid pΔEP0lac was linearized by *Eco*RI, and used for cotransfection with the purified viral DNA to produce recombinant virus.

rr (ribonucleotide reductase gene)-mutant

Mutant PRV was engineered by deleting the *ep0* and *rr* genes (Prv-rrep0lacgfp), which made the virus unable to replicate in neurons. The 5-kbp SalI-F fragment of PRV DNA containing the *rr* gene was isolated and cloned into pRL494, resulting in pRR. This plasmid was cleaved with ScaI and MluI restriction enzymes, resulting in the loss of a 1,805-bp sequence which includes a 1,789-bp fragment from the 3' end of *rrl* and a 7-bp sequence from the 5' end of *rr2*. EcoRI or HindIII linker was attached to the DNA ends filled up with Klenow enzyme, generating pRRI and pRRH, respectively. LacZ gene casette was subcloned to the EcoRI or HindIII site of the plasmid.

ASP (putative antisense promoter) region.

The *Bam*HI-8'-F PRV DNA fragment was isolated and subcloned into the pRL525 vector resulting in p525-B8'-F. The *Dra*I site of this PRV sequence was converted to *Eco*RI by linker insertion generating pASP-RI. Alternatively, *Bam*HI-8'-F fragment was subcloned into pRL479 to give p479-B8'-F, followed by conversion of the *Dra*I site to *Hind*III resulting in pASP-HIII. The various reporter gene expression cassettes (gfp, troponeon) were inserted either into the *Eco*RI site of pASP-RI or into the *Hind*III site of pASP-HIII, resulting in the generation of ASP-based targeting constructs.

Neut region

The *Bam*HI-8' segment of viral genome was subcloned to pRL494 plasmid vector. The unique *Stu*I restriction endonuclease site of *Bam*HI-8' fragment of PRV DNA was converted to *Eco*RI by linker insertion obtaining a plasmid termed pB8'-RI. We used pDsred2 (purchased from Clontech Laboratories) as the source of red fluorescent protein gene. The *Ase*I site at the 5'-end of hCMV promoter and the *Afl*II site at the 3'-end of

SV40 polyadenylation sequence were converted to *Eco*RI resulting in an expression cassette containing the DsRed2 gene and the regulatory sequences bracketed by *Eco*RI sites. This *Eco*RI fragment was subcloned to the *Eco*RI site of pB8'-RI, which resulted in the targeting vector, termed pNeut-DsRed. BaDsRed was linearized by HindIII enzyme and used to generate recombinant viruses by means of homologous recombination using standard techniques.

Transfection and selection of recombinant viruses

Reporter protein-encoding plasmids were transfected to PK-15 cells with Lipofectamine^{TM} transfection reagent (Invitrogen ^{TM}) according to manufacturer's instructions. Growing PK-15 cells were co-transfected with linearized targeting vector and viral DNA. Recombinants were selected on the basis of fluorescence/color of infected cells and were isolated by 6-15 cycles of plaque purification.

Recombinant PRV strains which were used during my work:

Name	**Genome**	**Marker gene**
Ka-gEI-Orange	Ka	Orange
Ba-DupmemTFP	Ba	TFP
Ka-VHSlac	Ka	LacZ
Ka-ep0lac	Ka	LacZ
Ka-VHSlac-gEIΔDupCFI	Ka	LacZ and CFP
Ba-DupGreen	Ba	GFP
Ba-NeutRed	Ba	dsRed
Prv-rrep0lacgfp	Ka	GFP and LacZ
Ti4-PRV08	Ka	TN-L15 and dsRed

RESULTS

Gene expression analyses

Expression kinetics of regulator genes of PRV using cycloheximide (CHX), PAA treatment and untreated samples

Traditionally, herpes virus genes are classified into distinct kinetic groups on the basis of the effects of protein and DNA synthesis inhibition on the gene expression [21]. To test the requirement of *de novo* protein synthesis for PRV mRNA production, PK-15 cells were untreated or treated with 100 μg/ml CHX prior to the infection of cells with the virus at a MOI of 0.1. At the indicated time points, RNA was isolated and converted to cDNA, which was subsequently analyzed by qRT^2-PCR. The degree of inhibition [$1\text{-}R_{i\text{-}CHX}$) × 100] was found to range between 97.3 and 100% for all but one protein encoding PRV gene (the *ie180* gene) and two antisense transcripts [the long-latency transcript-1 (LAT), antiparallel to *ep0*, and the long-latency transcript-2 (AST), antiparallel to *ie180*). IE180 mRNA displayed a significantly increased level of expression in the CHX-treated samples at the analyzed 3 time points: 2.27-fold at 2 h; 5.55-fold at 4 h; 1.4-fold at 6 h pi, the expression of IE180 mRNA was found to be significantly increased. We explain this phenomenon in that the IE180 proteins exert an inhibitory effect on their own synthesis (upon the binding of their own promoters), which is resolved by CHX blocking protein synthesis from the IE180 mRNAs. The other exception for the negative CHX effect is AST: a 1.72-fold increase at 2 h and a 3.983-fold increase at 4 h. Interestingly, at 6 h pi AST is significantly repressed by CHX. The repression of LAT expression at 2 h pi is relatively low, but this antisense transcript is significantly blocked by CHX at 4 and 6 h pi. Overall, our CHX analysis indicated that the only true IE protein-encoding gene of PRV is *ie180*. While the *icp27* and *icp0* genes in the HSV are IE genes, their PRV homologues *ul54* [11] and *ep0* [10] genes were earlier described as E genes, which was confirmed by our CHX analysis (Figure 1). In HSV, *icp22* gene is expressed with IE kinetics; our analysis revealed that its homologous counterpart, the us1 gene of PRV, is drastically blocked by CHX, and hence it is not an IE gene. As a result of CHX treatment, AST exhibits elevated levels at 2 and 4 h pi, which indicates that IE180 transcription factor exerts a negative effect on its expression. Thus, AST appears to be an IE transcript.

However, AST displays fairly low relative expression at 1 h pi, and therefore it cannot be regarded as an IE gene beyond doubt [21].

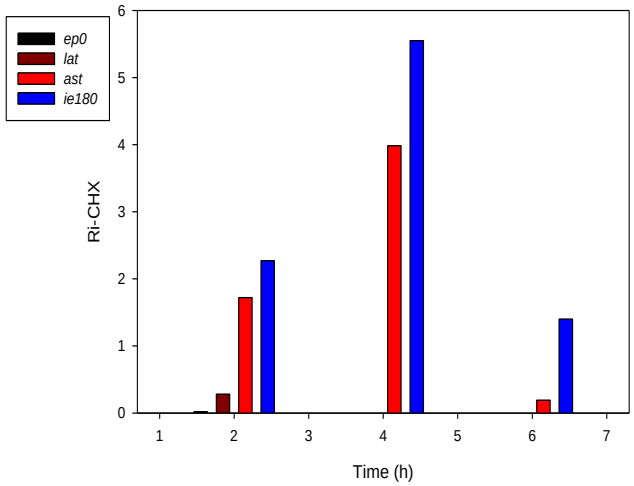


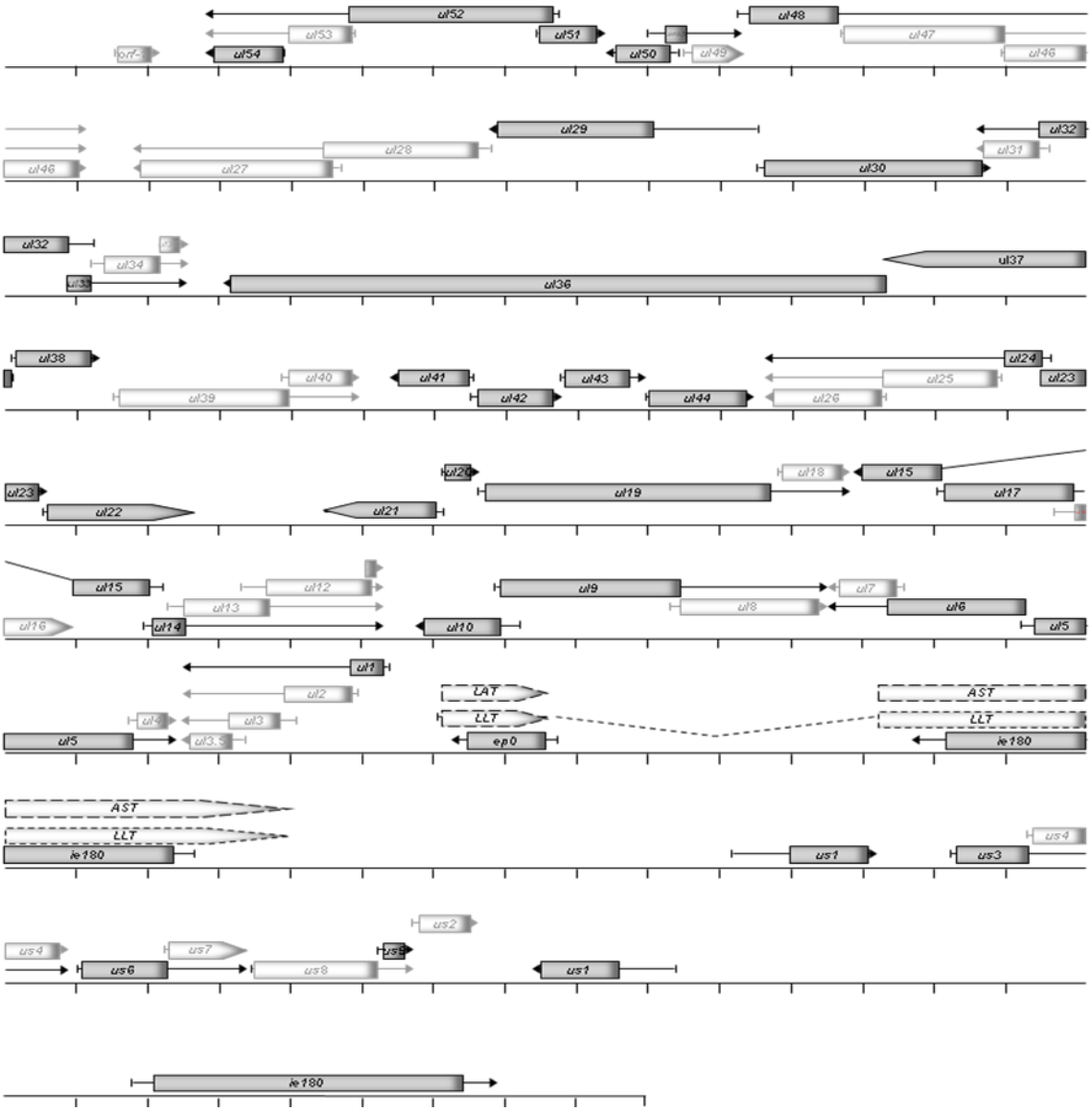
Figure 1.
The effect of CHX on the expressoi of ep0, LAT, ie180 and AST at 2h, and 6h pi .

For examination of the dependence of the PRV genes on DNA replication, PK-15 cells were infected with the virus (0,1 MOI) in the absence or presence of 400 μg/ml PAA, an inhibitor of DNA polymerase. It was expected that PAA would exert a more drastic effect on the L genes because the expressions of these genes are highly dependent on DNA replication. However, PAA must affect the E gene expression, too. The reason for this is that the gene expression is dependent not only on the promoter activity, but also on the copy numbers of the genes, which are higher after DNA replication than in the initial phase of infection when the PRV DNA is represented in a single copy in a cell (at least in our system). The 2 antisense transcripts displayed a surprising response to PAA treatment: the level of LAT increased to 2.94-fold at 4 h, and droped to 0.007-fold at 6 h pi relative to the untreated sample; while the level of AST increases close to 40-fold at 4 h, and 3-fold at 6 h pi. The *ie180* gene expression is also significantly inhibited by PAA (0.211); however, we found that *ie180* was expressed in an "irregular" manner in other analyses. We found, that the *ie180* and AST and LAT genes cannot be classified on the basis of the $R_{i\text{-}PAA}$ data alone, because they show unique expression kinetics. LAT and AST respond differently to CHX and PAA treatment, indicating that these antisense transcripts are, at least partially, under different regulation. We presume that the putative antisense promoter (ASP; [10]) controls the expression of AST [21].

Dependence of expression properties of 37 PRV genes on the multiplicity of infection

In this study, PK-15 cells were infected with pseudorabies virus at MOIs of 0.1 and 10 [83]. Albeit the difference in the infectious dose in the two parallel experiments was 100-fold, an individual cell was invaded by only 10 times more virus particles in the high-MOI than in the low-MOI experiment ($5x10^6$ versus $5x10^5$ infected cells), the reason for this being that in the latter case approximately 90% of the cells remained uninfected. Cells were harvested at 0, 1, 2, 4 and 6 h post-infection (pi), as in our earlier report [21]. We used 6 h as the maximum infection period in order to exclude the possibility of the initiation of new infection cycles in the low-MOI experiment. In this study, we analyzed the expression of 37 genes (53% of the total PRV genes) and two antisense transcripts (AST and LAT) (Figure 2) [83]. For the calculation of relative expression ratios (Rs), we used the average 6 h E^{Ct} values of the high-MOI experiments of both the "samples" and the "references" as controls. We used a correction factor of 10 for the calculation of R for the low-MOI experiment. With this calculation technique, approximately the same numbers of infected cells, and hence the relative amounts of transcripts in an average infected cell, were compared in the two experiments. However, in the high-MOI experiment, the proportion of the genome copy number in an infected cell was also 10-fold higher on average, at least before the start of viral DNA replication (the first 2 h pi), the reason for this being that in the high-MOI experiment 10 virus particles infected an average cell, while in the low-MOI infection 10 per cent of the cells were infected with a single virus particle. Thus, to compare the gene expressions from a single virus DNA per cell, two normalizations are necessary: multiplication of the R values of the low-MOI data by 10, and division of the R values of the high-MOI data by 10. In some calculations, the original data were handled accordingly (see the indications in the particular cases). The relationship between the infectious dose and the genome copy number of PRV becomes non-linear in later stages of viral infection; the DNA copy numbers in the two experimental situations are therefore not comparable on the basis of the infectious dose. The R values of LAT and AST were calculated by using the 6 h E^{Ct} values of the corresponding genes, *ep0* and *ie180*, respectively, as the reference gene [83].

Figure 2. *Localization of examined PRV genes on the viral genome. The direction of transcription is indicated by the arrows. Grey boxes indicate examined genes. Broken-line boxes show the known antisense transcripts of PRV. Unexamined genes are shown as white boxes.*



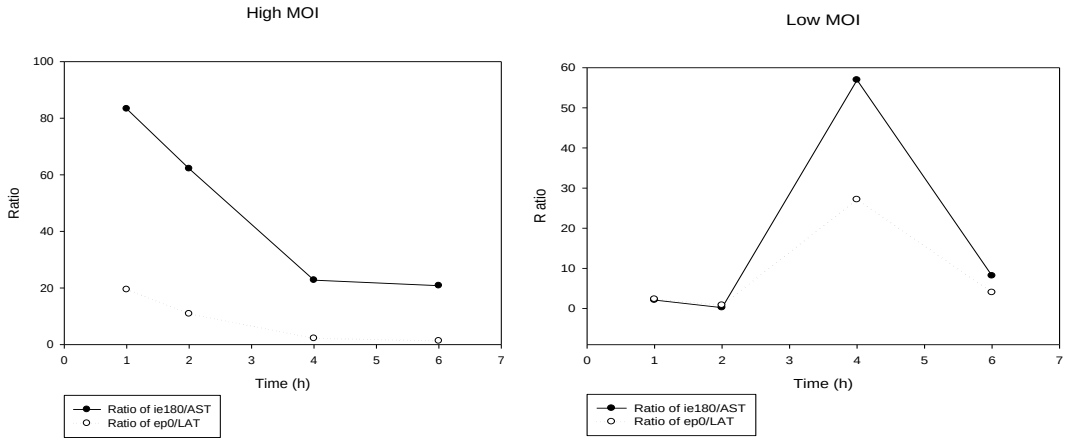
R_{Δ} values were used to monitor the net change in the quantity of viral transcripts within a given period of time. R_a shows the ratio of the changes in the amounts of transcripts between two adjacent time points. We considered two principles for the selection of genes for expression analyses [83]. (1) We analyzed the upstream genes of each nested gene cluster, the reason for this being that these genes are not overlapped by other genes,

and the amounts of these transcripts are therefore proportional to their protein products. This is in contrast with the downstream genes, which, if transcribed from the promoter of an upstream gene, are not translated, because they do not have cap sequences that are required for the recognition by the ribosomes. (2) Furthermore, we analyzed genes that are of primary importance in the regulation of global viral gene expression, such as *ie180, ep0, vhs* and *ul54*.

In the first 2 h of infection, the viral DNA replication has not yet been initiated, and the copy number of viral genomes in a cell therefore corresponds with the infectious dose. In this analysis, we found that the mRNA levels of most examined PRV genes were higher in the cells infected with the high MOI than in those infected with the low MOI at both 1 h and 2 h pi. This was not unexpected since in the former case viral DNAs were represented in an approximately 10-fold higher proportion in an average infected cell. Exceptions to this were the transcripts *ul1, ul33*, and *ul51* mRNAs at 1 h pi, and *ul36, ul38, ul43*, and *ul48* mRNAs at 2 h pi, and at both 1 h and 2 h: *ie180* and *ul30* mRNAs, as well as, LAT and AST. However, the expression levels normalized to the genome copy number (i.e. using R/10 values in the high-MOI infection) showed an inverse pattern: only a few genes were expressed at higher abundance in the high-MOI than in low-MOI infection. AST was expressed at a considerably higher quantity in the cells infected with the low MOI than in those infected with the high MOI. In the high-dose infection 6 of the 37 genes (*ie180, ul36, ul50, ul54, us1*, and *ul24*) exhibited higher expression levels at 1 h than at 2 h pi. It should be noted that 3 of them (*ie180, us1* and *ul54*) are regulatory genes. The fourth regulatory PRV gene, *ep0,* is expressed at a very high level during the first 2 h in the high-MOI infection ($R_{1\ h}$=1.87, $R_{2\ h}$=2.05). Apart from *ep0, ul5* was the only gene that was expressed at a higher extent in the early stages of infection than at 6 h pi in the high-MOI experiment. The *ie180* gene is the only one that was expressed in a higher amount at 1 h than at 2 h pi under both experimental conditions. Overall, it appears that the 4 regulatory genes were expressed at relatively high levels before the onset of DNA replication in the high-MOI infection, which was not the case in low-MOI infection, with the exception of the *ie180* gene. We think that the reason for the higher expression of regulatory genes at the onset of viral DNA replication in the high-MOI infection is that more regulatory proteins are needed to carry out the multiplication of a

higher copy number of the viral genome. The rate of change in gene expression within the 1 h to 2 h interval (R_{2h}/R_{1h}) was higher in more than two-thirds of the PRV genes (25/37) in the low-MOI than in the high-MOI infection. The proportion of AST to *ie180* mRNA molecules (R_{AST}/R_{ie180}) was 0.47 at 1 h pi, and 4.72 at 2 h pi in cells infected with the low MOI, while this ratio was extremely low (~0.01) at both 1 h and 2 h pi in the high-MOI infection (these data are only semi-quantitative since the primer efficiencies in the RT reaction are not necessarily equal for the two transcripts). Thus, the proportion of AST to *ie180* mRNA [($R_{AST\text{-}low\ MOI}/R_{ie\text{-}low\ MOI}$) / ($R_{AST\text{-}high\ MOI}$)/$R_{ie\text{-}high\ MOI}$)] was higher at 1 h pi and 2 h pi in the low-MOI than in the high-MOI infection. The *ep0* gene is expressed in higher quantity at both 1 h pi and 2 h pi in the high-MOI infection than in low-MOI infection, which is in contrast with LAT, its antisense partner, whose expression level was lower in the high-MOI infection. Thus, the ratios of LAT to *ep0* mRNA molecules were 8.33-fold higher at 1 h pi and 13.05-fold higher at 2 h pi in the low-MOI than in the high-MOI experiment, although, unlike AST, LAT is abundantly expressed in the high-MOI infection (Figure 3) [83].

Figure 3. *The ratio of ie180 and ep0 mRNAs to their antisense partners*
The continuous lines illustrate the ratio of ie180 mRNA to AST, while the dotted lines represent the ratio of ep0 mRNA to LAT at the low- and high-MOI infections.



At 4 h pi the transcript levels of more than three-quarters of the PRV genes (28/37) were still higher in the cells infected with the high MOI than in those infected with the low

MOI. However, in about two-third of the viral genes the rate of change (R_a values) in the expression level was higher in the low-MOI than in the high-MOI infection [24/37 within the 2 h to 4 h period, and 25/37 within the 1 h to 4 h period]. In the low-MOI infection, the amounts of 5 transcripts (*ul5*, *ul44*, *us1* and *us6*) were less than 10% of those in the high-MOI infection at 4 h pi. All of the examined *us* genes are expressed at a significantly lower level in the low- than in the high-titer infection at 4 h pi. There were significant decreases in the quantities of both AST and LAT in the low-titer infection at 4 h pi relative to the 2 h values. We explain this phenomenon by the negative effect of the regulatory genes on their antisense partners. Regulatory genes are upregulated at the onset of DNA replication (in order to facilitate this process), which exerts an inhibitory effect on the expression of AST and LAT. In contrast, there were increases in the amounts of antisense transcripts in the high-MOI in this time interval. However, while LAT was expressed at high level under the high-MOI conditions, the AST expression remained extremely low in this period of infection. The amount of the *ie180* transcript was practically unchanged within the 2 h to 4 h infection period under either infection conditions. There was a 4.7-fold increase in the *ep0* mRNA level within the 2 h to 4 h infection period (R_{4h}/R_{2h}) in the low-MOI infection, as compared with only 1.4 in the high-MOI experiment. On average, the amounts of mRNAs in low titer infection became higher than those in the high-infection titer by 6 h pi in more than half of the PRV genes (22/37). We assume that the reason for this might be that the *ie180* gene, the major coordinator of gene expression, is expressed at higher levels at 4 and 6 h pi at low-MOI than at high-MOI infection. Moreover, in the high-MOI infection the amount of AST reached almost 30% of the transcript level in the low-MOI infection, while LAT was expressed at approximately the same level under the two infection conditions at 6 h pi. The genes expressed at lower levels in the low-dose infection appeared to be clustered on adjacent genomic locations (Fig. 2). Each gene and the two antisense transcripts were expressed at higher rates (R_a values) within the 4 h to 6 h period in the low-MOI than in the high-MOI infection without exception. In the high-MOI infection, 11 genes and LAT peaked at 4 h within the 6-h examination period, while in the low-MOI infection only the *us3* transcript had a slightly lower R value at 6 h than at 4 h pi. The *us3* gene was the only one among the 70 PRV genes which was expressed at a higher level at 4 h than at 6 h pi

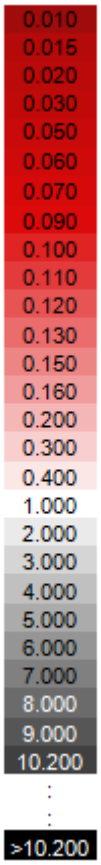
in another study [84]. Intriguingly, the *ep0* mRNAs reached a 3.5-fold higher level in the low-dose than in the high-dose infection in an average cell at 6 h pi. Furthermore, at 6 h pi the *ul1* and *ul51* genes were expressed at an approximately 10 times higher level under the low-MOI than under the high-MOI conditions [83].

Most genes were expressed at a lower level in a cell in the low-MOI experiment in the first 4 h of infection, but more than half of these gene products surpassed the high-MOI values by 6 h pi. The R values of 3 PRV genes (*ie180, ul1 and ul30*) were higher in the low-MOI than in the high-MOI infection at every examined time point, while the opposite was true (the R values of high-MOI were always higher) in 13 genes: *ul5, ul15,ul17,ul19, ul23, ul24*, *ul44, ul49.5, ul54*, *us6, us9, us1 and us3* (Fig. 4). These latter genes form clusters on the basis of their localization on the genome (genes in close vicinity are underlined), which suggests that the adjacent genomic sequences might be under common regulatory control. This observation is supported by the similarity of the R_a curves of adjacent genes [83]. For example, the expression rates of the *ul36, ul37* and *ul38* genes were similar to each other in both experiments, but each of them exhibited an inverse expression pattern in the two infection conditions. All genes were expressed at a higher rate (R_a) within the 1 h to 6 h period of infection in the low-titer experiment, except for *ie180* and the two antisense transcripts. The quantities of *ie180* mRNAs were similar in the two experiments, except at 1 h pi, where the level of the transcripts was 2.8-fold higher in the low-MOI infection. Thus, the amount of total *ie180* transcript in an infected cell appears to be under strict control, independently of the initial infection conditions. In contrast, the expression of the *ep0* gene differed basically in the two experiments. In the high-MOI experiment, the amount of *ep0* mRNAs was high from the first hour of infection, and its expression even declined by 6 h in the high-MOI infection, while the amount of these transcripts rapidly increased throughout the 6-h infection period in the low-dose infection, and reached a 3.5-fold higher level compared to that of the high-dose infection by 6 h. The ratio of sense and antisense transcripts during the 6-h infection period displayed intriguing patterns. First of all, in the high-MOI infection the amount of AST and its ratio to *ie180* mRNA were very low throughout the 6-h infection period. We demonstrated an inverse relationship in the expression kinetics of *ie180* mRNA and AST and also *ep0* mRNA and LAT in the low-MOI infection; however, we

did not observe this inverse relationship between the complementary transcripts under the high-MOI conditions.

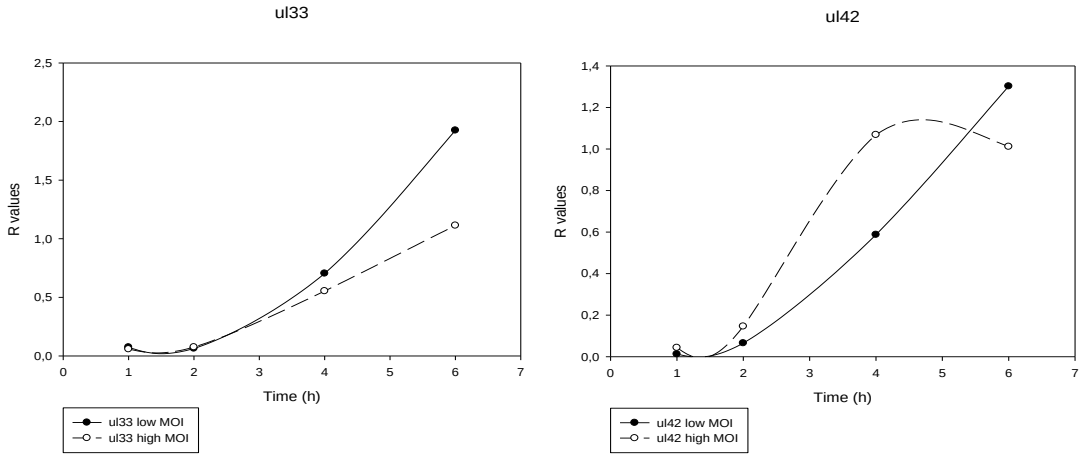
Figure 4. *Heatmap-like representation of the ratio of transcripts produced in the low-MOI and high MOI infection ($R_{t\ low\ MOI}/R_{t\ high\ MOI}$) PK-15 cells were infected with the PRV-Ka strain t different MOIs (0.1 and 10). Real-Time PCR data were normalized to 28S RNAs. The R_{low}/R_{high} values are plotted in a heat map-like manner. Black boxes indicate the highest ratio, and dark-red boxes the lowest values. White boxes demonstrate approximately equal values.*

Gene (Kinetic class)	1h/1h	2h/2h	4h/4h	6h/6h
ul54 (Early)	0.135	0.396	0.436	0.676
ul52 (Early)	0.088	0.322	1.777	3.785
ul50 (Early)	0.164	0.947	0.951	1.638
ul29 (Early)	0.428	0.452	0.749	1.006
ul30 (Early)	1.671	1.746	1.253	3.185
ul23 (Early)	0.152	0.122	0.211	0.398
ul21 (Early)	0.801	0.834	1.001	1.252
ul9 (Early)	0.296	0.686	0.679	1.089
ep0 (Early)	0.237	0.411	1.363	3.490
us3 (Early)	0.093	0.117	0.210	0.231
AST (Early)	110.821	297.500	0.448	3.389
ul43 (Early late)	0.654	1.921	1.087	1.532
ul20 (Early late)	0.236	0.210	0.717	2.112
ul15 (Early late)	0.052	0.139	0.358	0.739
ul14 (Early late)	0.501	0.809	0.919	1.803
ie180 (Early late)	2.816	1.014	1.119	1.325
us6 (Early late)	0.130	0.158	0.090	0.199
us9 (Early late)	0.218	0.345	0.246	0.768
ul51 (Late)	1.958	0.061	2.004	10.134
ul49,5 (Late)	0.024	0.290	0.344	0.730
ul48 (Late)	0.477	1.569	0.431	0.986
ul32 (Late)	0.260	0.677	0.587	2.239
ul33 (Late)	1.275	0.842	1.271	1.729
ul36 (Late)	0.588	2.406	0.431	0.900
ul37 (Late)	0.171	0.621	0.341	1.541
ul38 (Late)	0.757	2.003	0.164	1.799
ul41 (Late)	0.052	0.341	0.623	1.248
ul42 (Late)	0.291	0.448	0.550	1.288
ul44 (Late)	0.127	0.023	0.075	0.222
ul24 (Late)	0.202	0.253	0.101	0.724
ul22 (Late)	0.419	0.354	0.689	1.077
ul19 (Late)	0.067	0.270	0.229	0.570
ul17 (Late)	0.080	0.024	0.173	0.938
ul10 (Late)	0.411	0.118	0.220	1.058
ul6 (Late)	0.074	0.055	0.412	1.249
ul5 (Late)	0.017	0.013	0.031	0.421
ul1 (Late)	2.017	1.179	1.532	8.854
us1(Late)	0.168	0.679	0.077	0.563
LAT (Late)	1.973	5.371	0.078	1.122



In our earlier report [21], we showed that treatment of infected cells with cycloheximide (a protein synthesis blocker) resulted in significant increases in the amounts of both *ie180* mRNA and AST, while phosphonoacetic acid (a DNA synthesis inhibitor) treatment led to a decrease in *ie180* mRNA and a significant increase in the AST level. These results suggest a negative effect of the IE180 transactivator on ASP synthesis. We explain the huge drop in ASP level in the infected cells in the early stage of the high-MOI infection by the presence of a 10-fold higher amount of inhibitory IE180 protein localized in the tegument of the infecting virions [85]. The same reason could account for the lower *ie180* mRNA level in the high-MOI infection. The *us1* gene was expressed in the late kinetics in our earlier low-MOI analysis in both phophonoacetic acid-treated and non-treated samples. These results are in concordance with those of the present high-dose infection experiment, i.e. *us1* mRNA was expressed at a relatively low level at 1 h, which even dropped by 2 h pi. The highest rate of *us1* mRNA expression was observed at 4 h, with a rate ($R_{4\ h/2\ h} = 13.32$) typical of L genes. The Pearson correlation coefficients of the R, R_{Δ}, and R_a values precisely show the degree of similarity (or differences) of the expression kinetics of the genes in the low- and high-MOI experiments (Figure 5). Several genes exhibited high correlations for all three parameters. For example, the *ie180*, *ul19*, *ul21*, *ul22*, *ul42* and *ul43* genes gave high correlation coefficients for the R, R_{Δ} and R_a values. The *us1* gene behaved in an irregular manner; it gave a relatively high correlation for the R values, no correlation of R_{Δ}, and an inverse correlation for the R_a values. AST yielded relatively high negative values for all three parameters, indicating a significant negative correlation. The expressions of LAT under the two experimental conditions did not correlate on the basis of the R values, whereas it gave a very high negative correlation for its R_{Δ} and R_a values [83].

Figure 5. *Typical expression curves. These pictures show the expression dynamics of two genes in the low-MOI and high-MOI infection.*



Effect of vhs-knock out virus strain on the expression of other PRV genes

VHS molecules modify the transcript levels of each individual gene in a varying manner, but we have observed important regularities of these effects typical to the different kinetics classes of genes (see below). Data show that all of the examined PRV transcripts are expressed at a lower level from the *vhsΔ* genome than from that of wild-type's ($R_r = R_{\Delta vhs}/R_{wt} < 1$) at 1h pi (Figure 6). Our explanation for this phenomenon is as follows. Although, viral mRNAs are also the targets of VHS protein, the preferential degradation of cellular mRNAs by the viral RNase at this stage of infection might compensate this effect in two different ways: by the elimination of competition between the host and the virus for the translation apparatus of the cell in favor of the virus, and by the production of new ribonucleotides serving as raw materials for the newly synthesized viral transcripts. We have found no bias of VHS effect toward any kinetic classes of PRV genes at this time of infection. At 2 h pi tegument VHS proteins exert an inhibitory effect on the accumulation of PRV transcripts in all of the three kinetic classes (E, E/L and L; Figure 6) with a few exception. Thus, tegument-derived VHS proteins appear to switch their inhibitory effect toward viral mRNAs, but without distinction between the kinetic classes of viral transcripts within the 1 to 2 h period of infection [86]. There is an overall fall of VHS effect in the 2 to 4 h infection period (Figure 7) ($\overline{R_r} = \overline{R}_{\Delta vhs} / \overline{R}_{wt}$).The gene expression curves show that the average transcript levels of E/L and L genes in *vhs* null mutant become practically the same as in the *wt* virus at 4 h pi. This is in contrast to the E transcripts whose average levels remain higher in *vhs* mutant strain than in *wt* virus,

but their levels become also somewhat lower at 4h than at 2h pi in the mutant PRV. We explain this phenomenon by the dampening level of tegument VHS proteins, which uniformly acts on the different kinetic classes of viral genes and the raising level of newly synthesized (*de novo*) VHS proteins, which appear to lower the levels of E transcripts in a differential manner. Expressions of early PRV genes tend to be significantly higher in *vhs*-knockout than in *wt* virus from 4 to 24 h pi, which is most remarkable at time points 8 and 12 hours of infection (Figures 6 and 7).

Figure 6. *Heatmap-like presentation of the ratio of transcripts produced in vhs-mutant and wild-type virus infection* ($R_r = R_{\Delta vhs}/R_{wt}$) *Black boxes indicate the highest ratio, and red boxes the lowest values. Light-grey boxes demonstrate approximately equal values*

K	Gene	1h	2h	4h	6h	8h	12h	18h	24h
IE	*ie180*	0,529	0,951	0,849	1,346	3,172	3,379	2,007	1,580
E	*ep0*	0,400	1,184	1,871	5,192	6,064	11,070	3,749	3,532
E	*ul9*	0,398	1,582	1,087	1,111	1,586	1,999	0,976	1,067
E	*ul21*	0,779	3,144	1,779	2,393	2,629	5,605	1,823	2,400
E	*ul23*	0,665	1,219	0,827	1,988	4,876	3,886	1,745	2,969
E	*ul29*	0,567	1,743	1,331	1,754	5,507	3,306	1,348	1,196
E	*ul30*	0,646	4,154	2,277	1,835	3,660	3,987	1,627	1,147
E	*ul50*	0,385	3,337	1,038	1,829	4,614	2,509	3,517	1,933
E	*ul54*	0,294	1,893	0,917	1,774	3,986	3,566	1,836	2,633
E	*ul52*	0,505	2,071	1,957	2,111	4,142	2,585	1,121	0,541
E	*us3*	0,789	0,603	1,207	1,581	2,102	3,990	3,444	2,304
E/L	*ul14*	0,440	1,926	0,709	1,493	3,019	2,242	1,333	1,197
E/L	*ul15*	0,542	1,026	0,649	0,876	1,507	2,790	1,160	1,280
E/L	*ul20*	0,267	1,290	1,337	0,838	3,631	1,446	2,466	1,506
E/L	*ul43*	0,613	1,637	0,922	1,857	3,271	3,094	2,779	1,152
E/L	*ul53*	0,747	1,088	1,074	2,239	4,548	1,337	1,798	0,967
E/L	*us6*	0,418	3,170	0,301	0,531	0,527	0,675	0,697	0,908
E/L	*us9*	0,533	2,785	0,687	0,956	1,508	1,881	1,969	1,206
L	*ul1*	0,409	2,196	0,515	0,691	0,889	1,286	0,774	1,122
L	*ul5*	0,380	0,516	0,285	2,340	1,264	3,300	0,557	1,477
L	*ul6*	0,579	0,488	0,703	1,147	1,240	1,685	1,674	1,241
L	*ul10*	0,512	1,993	0,988	1,269	1,017	0,683	0,874	0,578
L	*ul17*	0,523	0,912	1,303	1,526	1,410	1,754	1,377	1,173
L	*ul19*	0,350	2,060	0,700	1,021	1,150	1,562	0,310	0,261
L	*ul22*	0,837	2,176	1,987	0,874	3,065	1,530	2,274	2,032
L	*ul24*	0,518	4,899	0,848	1,655	2,360	2,051	0,971	1,179
L	*ul32*	0,945	2,390	0,971	1,613	1,199	1,247	0,965	1,024
L	*ul33*	0,535	4,466	1,466	1,167	2,023	1,456	1,388	1,171
L	*ul36*	0,463	3,598	1,567	1,812	1,708	1,043	0,867	0,837
L	*ul37*	0,390	2,683	0,952	1,588	2,594	2,291	0,666	0,670
L	*ul42*	0,498	1,284	0,562	0,869	0,781	1,239	0,865	1,561
L	*ul44*	0,250	1,396	0,397	0,375	0,566	0,446	1,141	0,650
L	*ul48*	0,362	1,881	0,646	0,679	1,059	0,976	0,857	0,638
L	*ul49,5*	0,199	1,763	0,721	0,753	0,690	1,557	1,329	0,755
L	*ul51*	0,474	1,440	1,047	4,190	2,367	1,650	1,107	1,473
L	*us1*	0,250	0,941	0,645	0,815	0,752	2,333	2,035	1,191
	LAT	0,288	1,260	0,409	0,776	1,087	1,229	1,176	0,997
	AST	0,376	2,553	0,415	0,943	1,143	2,437	0,787	0,651

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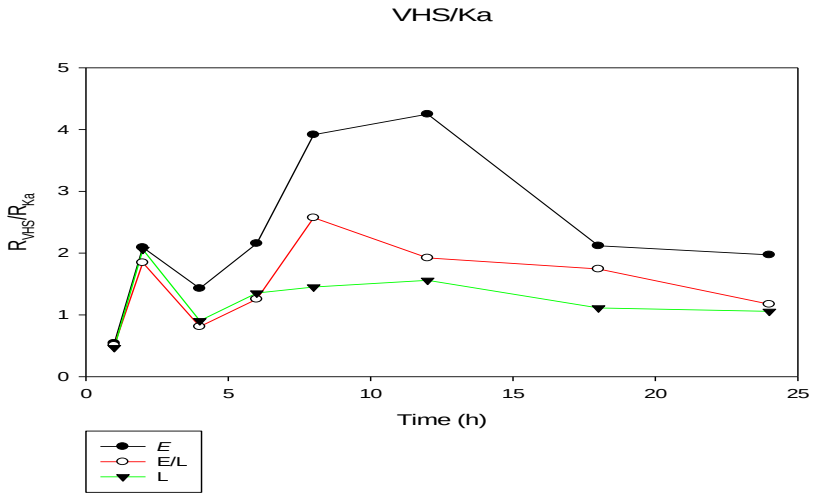
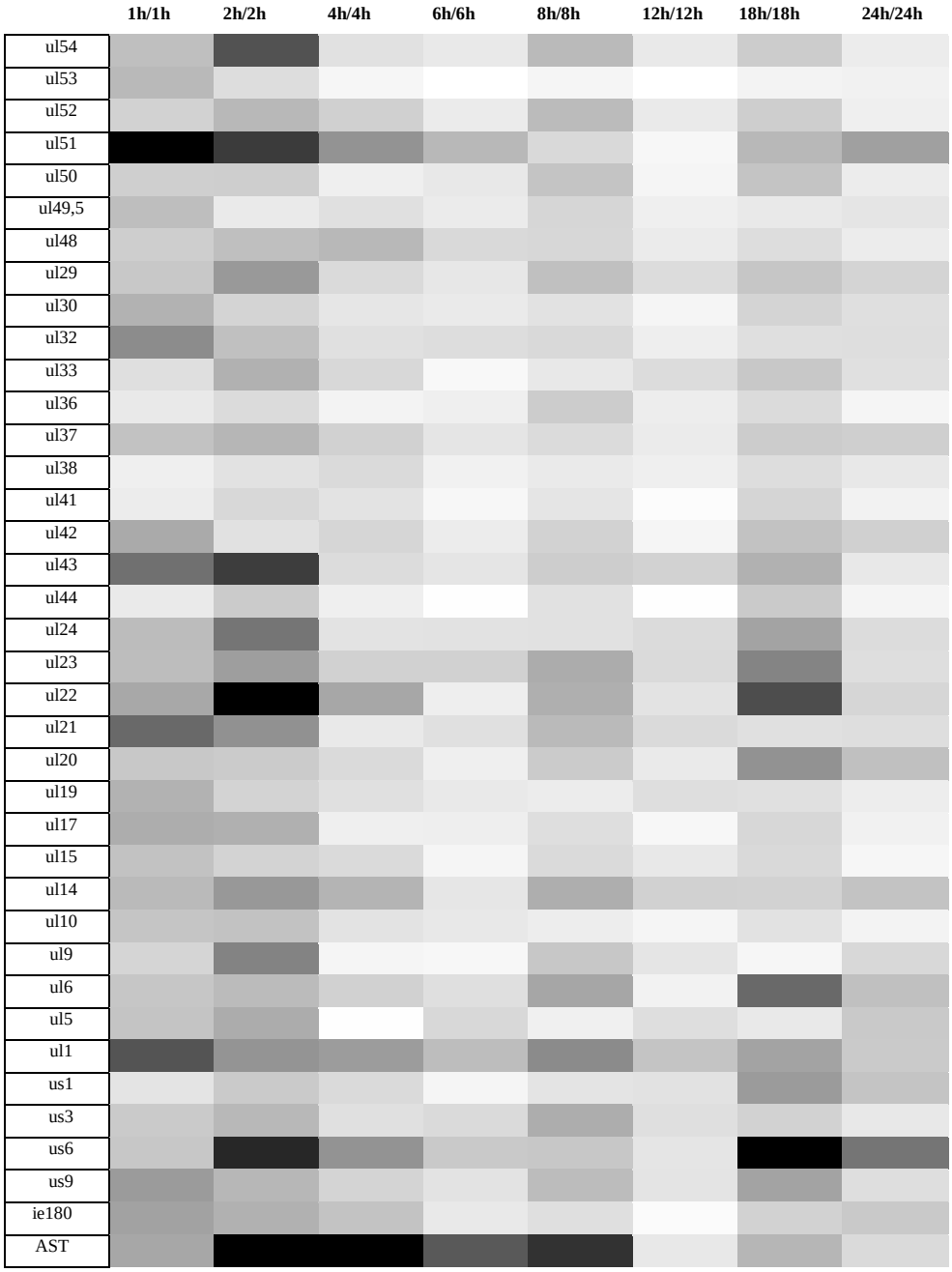
Figure 7. This figure shows that the VHS protein exerts its strongest effect on E and E/L genes between 8 and 12 h pi.



Average late gene expressions have not been significantly changed in the mutant virus compared to the *wt* virus after 4h of infection. There are some L genes whose transcripts levels are even lower in the *vhsΔ* background than in the wild-type's at many time points of infection; for example, *ul1*, *ul5*, *ul10*, *ul19*, *ul44*, *ul48*, and *us1* genes. The average levels of E/L transcripts always fall between E and L in the 6 h to 24 h pi interval. No selective effect of VHS activity is observed in E/L and L kinetic classes of PRV genes at 4 and 6 h time points of infection. In fact, the average effect of VHS protein is similarly low on E/L and L transcripts at the first 6 h of infection, while this is not the case in E genes of the virus (Fig 7). The $\overline{R_r}$ value of E genes rapidly increases within the 4 h to 12 h period but sharply drops by 18 h pi; however, the amount of these transcripts remains higher in *vhs*-deleted virus than in *wt* virus even at this time point (18 h) of infection. The $\overline{R_r}$ value of E/L transcripts becomes larger than 1 from at each time points between 6 to 18 h peaking at 8 h pi at a medium level compared to E mRNAs. According to our date, there is no selective effect of VHS protein on E/L transcripts at 24 h pi. Transcripts of L genes are only slightly affected by the *de novo* VHS proteins within the 6 h to 24 h interval of infection with a peak of R_r = 1.56 at 12 h pi. Examination of individual E genes reveals that their maximal expressions are shifted from 4 and 6 hours to 8 and 12 hours in most genes (Figure 7). Furthermore, while in the *wt* virus the average R values of L genes are higher than those of E genes; it is basically changed in favor of E genes in

vhsΔ. Together, the absence of VHS protein renders the kinetics of early gene expressions similar to those of late genes'. These results suggest a critical role of VHS protein in the transition of early to late phase transition of PRV gene expression. Our data show that VHS protein exerts far the highest impact on the transcript level of early protein 0 gene (*ep0*) among all of the examined 38 PRV transcripts (Fig 6). At the first hour of infection, similarly to other genes, VHS protein exerts a strong stimulatory effect on the transcription of *ep0* gene. Later on, VHS protein acts to lower the copy number of EP0 mRNAs, which culminates at 12 h pi, where the level of EP0 transcripts become more than 11 times higher in *vhs* null mutant than in the *wt* virus. Furthermore, *ep0* transcription kinetics becomes highly correlated with that of *ie180* in the *vhs* null mutant virus (r = 0.858), which is not the case at all in *wt* virus, where there is no correlation in the expression kinetics between the two transcription factor genes (r = 0.210). Furthermore, in the *vhsΔ* strain there is a high correlation between EP0 transcripts and other PRV gene products (r = 0.868 on average), while there is an inverse correlation between EP0 mRNA and other PRV transcripts (r = - 0.571) in the *wt* virus. It cannot be excluded that VHS exerts its effect on the level of PRV transcripts through the regulation of EP0 mRNAs. According to this scenario, VHS proteins act to lower EP0 protein level by the degradation of EP0 mRNAs, which in turn, results in the selective lowering of E transcripts. Indeed, EP0 mRNA reaches its maximum at 4 h pi in the *wt* virus, while it peaks at 12 h at a very high level in *vhsΔ* virus. This delay in the expression maximum in *ep0* gene might account for the same delay of maximal expression of early genes. Furthermore, the drop of EP0 transcript level from 4 h pi might explain the decline in the speed of production of E gene transcripts in the *wt* virus. Furthermore, the similar transcription kinetics of IE180 and EP0 transcripts in *vhsΔ* virus might account for both the synchronized viral gene expression and the high correlation of transcript levels of PRV genes with IE180 transcript level in the *vhs* mutant virus. For explanation, in contrast to the *vhsΔ* strain, in *wt* virus the *ep0* gene is expressed in a kinetic different from that of *ie180* gene, which might disrupt the correlation between the amount of IE180 protein and the rest of PRV gene products. We analyzed the effect of *ep0*-knock out mutant virus strain on the expression of 37 PRV genes (Figure 9.).

Figure 9. *Heatmap-like presentation of the ratio of transcripts produced in ep0-mutant and wild-type virus infection (R_{tep0}/R_{tKa}) Black boxes indicate the highest ratio, and white boxes the lowest values. Light-grey boxes demonstrate approximately equal values*



PRV as a neural circuit tracer

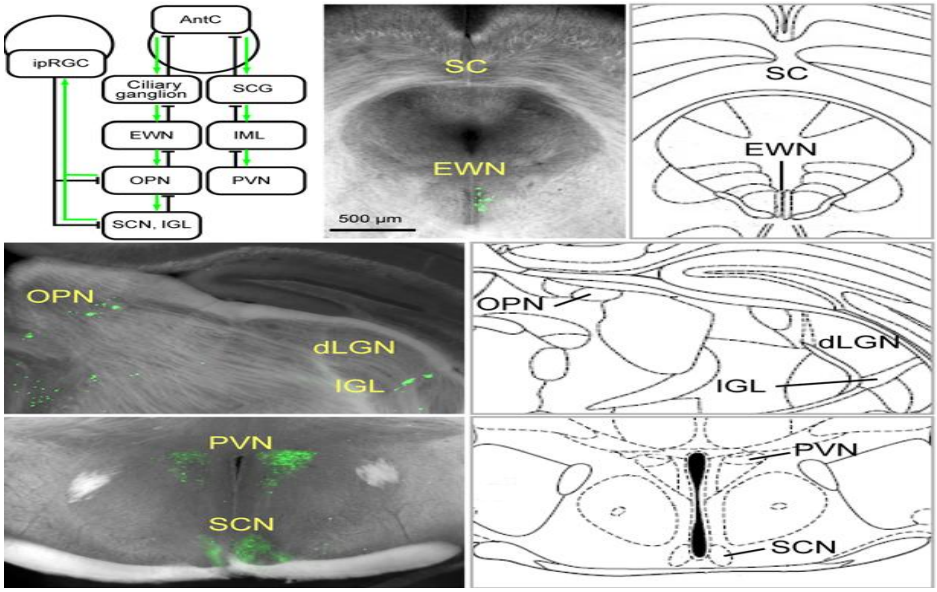
The following projects were done in collaboration with other research groups.

I focus on the results obtained in these projects more concisely than the result of kinetics expression studies, which were my main projects.

Retrograde spread (gE and gI deletion)

Recombinant PRVs were engineered (either derivatives of the Bartha (Ba) strain or the wild-type Kaplan strain) to be retrograde by deleting the *gI* and *gE* genes of the virus and named as As1-PRV08. To determine if As1-PRV08 is a retrograde transsynaptic tracer, we compared its spreading properties to the well characterized GFP-expressing retrograde PRV152 strain (recombinant Bartha strain). We injected As1- PRV08 into either the anterior chamber of the right eye or into the primary visual cortex (V1) of mice and tested putative retrogradely labeled retinal ganglion cells for TN-L15 expression in the contralateral eye. To reach these ganglion cells, the virus would need to travel retrogradely through at least one (from V1 through the lateral geniculate nucleus) or at least three (from the anterior chamber via the pupillary reflex pathway) synapses (Figure 10). We could conclude that PRV mutants with deleted gE and/ gI genes exhibit an exclusively retrograde transsynaptic spread [4].

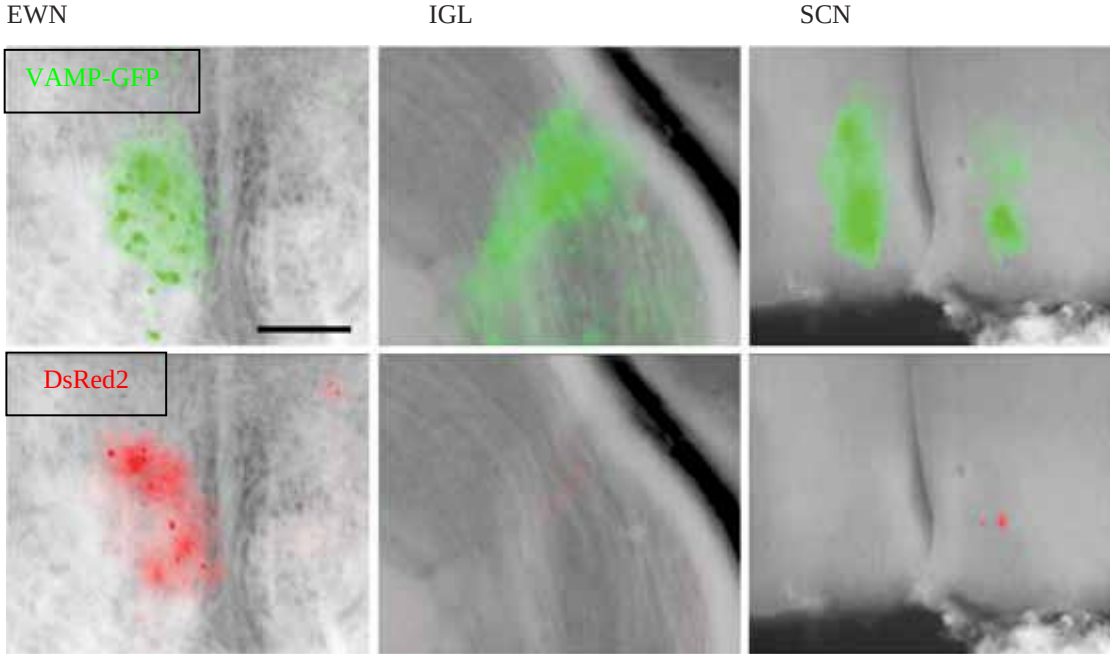
Figure 10. *Schematic diagram of the pupillary reflex pathway. Green arrows indicate a retrograde route along the reflex pathway (top, left, SCG: superior cervical ganglion, IML: intermediolateral nucleus of the thoracic spinal cord, PVN: paraventricular nucleus). Labeled brain areas after As1- PRV08 injection into the AntC of the right eye.*



Combined viruses

Our research group engineered PRV viruses, called timer PRVs (Ti-PRVs), that express two differently colored fluorescent proteins (GFP and DsRed2, with different maturation) which are detectable in a time shifted manner: membrane-targeted green fluorescence appeared at the early stage of infection (primary fluorescent protein), while the soluble red reporter was detectable several hours later (secondary fluorescent protein). Another type of genetically modified viruses is the Activity sensor viruses. To test whether the delay between red and green reporter expression was preserved in vivo, we injected Ti1-PRV07 (expressing membrane bound GFP and DsRed2) into the anterior chamber of the right eye in mice and analyzed the brain nuclei of the pupillary reflex pathways 3.5 d after infection (Fig. 11). In the Eddinger-Westphal nucleus (EWN), which is the first station of the viral spread in the brain, the expression of GFP and DsRed2 was similar. In contrast to the EWN, GFP levels were higher compared to DsRed2 in the olivary pretectal nucleus (OPN), intergeniculate leaflet (IGL) and the suprachiasmatic nucleus (SCN), which are one or two steps further in the retrograde route of the virus. These results confirmed that GFP can be detected earlier than DsRed2 in vivo [4].

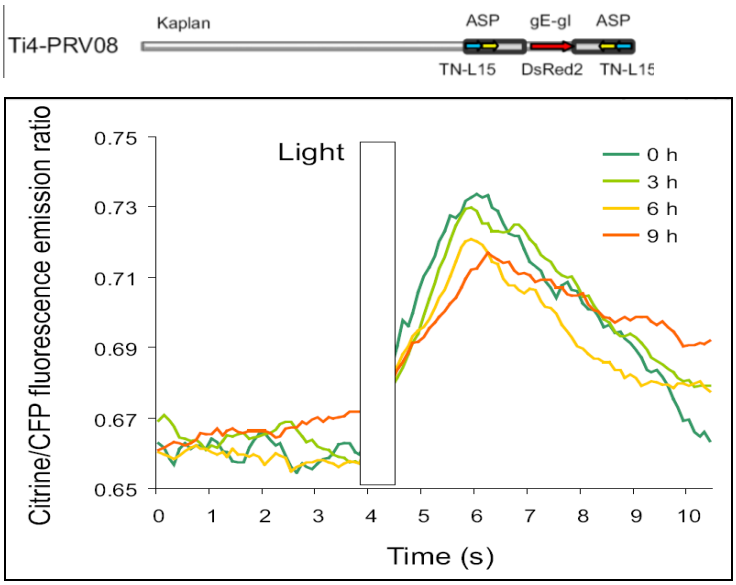
Figure 11. *Timer-PRVs. Green:red fluorescence emission ratio in different brain regions 3.5 d after Ti1-PRV07 was injected into the anterior chamber of the mouse right eye.*



Calcium plays an essential role in the function of nerve cells, therefore real-time monitoring of its concentration gives us information about the actual cell activity. TN-L15 is a calcium-sensitive chimeric protein which contains a calcium-binding domain, which is sandwiched between a pair of fluorescent proteins, a blue CFP and a yellow Citrine. The operation of this molecule is based on the Fluorescence Resonance Energy Transfer (FRET) that means CFP in its excited state may transfer energy to Citrine in case of close proximity. If we excite CFP when the calcium content is low, we see blue color. However in high calcium content FRET accrues. We can measure the Ca concentration on the basis of the ratio of blue and yellow.

To show that the physiological state of the infected cells didn't significantly change during the seven-hour period, we engineered PRVs with both Activity sensor and Timer functions. Four days after virus injection, light-evoked Calcium changes were monitored from ganglion cells by FRET. The recordings were repeated every hour until the appearance of the red fluorescent. Responses were similar in the green-to-red time window. This result suggests that until the cell turns red, electrophysiological measurements can be carried out (Figure 12) [88].

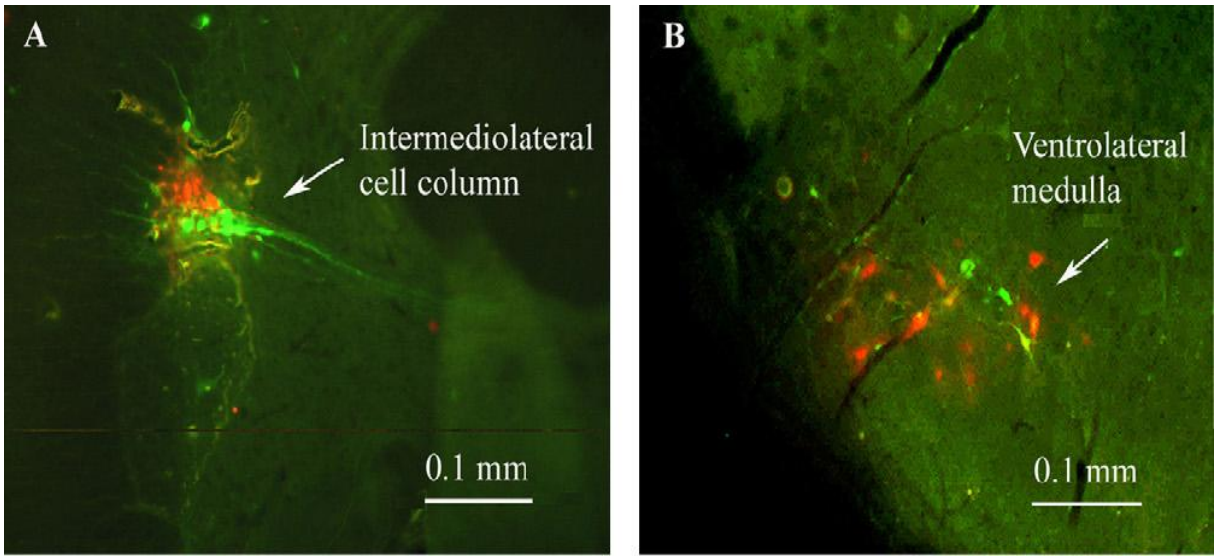
Figure 12. *Combined viruses. Genome of the Ti4-PRV08 and a plot of light-induced Ca2+ responses measured with Ti4-PRV08 from an ipRGC during 9 h after removing the retina from the left eye of the mouse.*



Dual-tracing

We have developed Ba-PRV based mutant viruses (Ba-NeutRed) via inserting dsRed fluorescent protein gene into the Neut region of PRV. In combination with Ba-DupGreen, Ba-NeutRed is a useful tool for transneuronal tracing. Following administration of neurotropic virus into the adrenal gland or into the ovary, double-labeled neurons could be observed in the ventrolateral medulla, the nucleus of the solitary tract, the caudal raphe nuclei, the A5 catecholaminergic cell groups, and in the hypothalamic paraventricular nucleus (Figure 13) [80].

Figure 13. *Dual-labeling. Coronal sections of rat brains. The left ovary was injected with pseudorabies virus expressing green fluorescent protein, while the left adrenal was inoculated with a recombinant strain expressing DS-RED. Neurons connected with the left adrenal gland or the left ovary are well distinguished.*

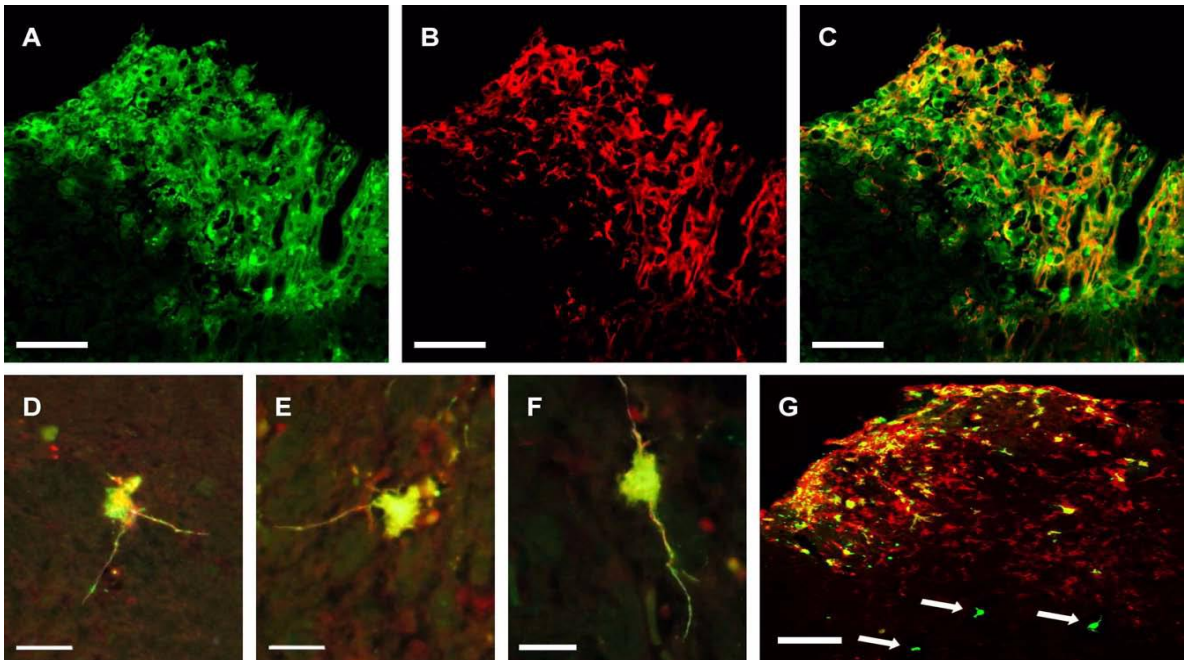


Investigating PRVs in human embryonic cord neurons

The aim of the present study was to investigate the efficacy of the genetically engineered PRV the Prv-rrep0lacgfp, in infecting human embryonic spinal cord neurons *ex vivo* and in establishing gene expression after transplantation (Figure 14). For this we generated a

triple-deletion mutant virus. This recombinant virus was constructed in two steps. The small subunit of the ribonucleotide reductase (rr) gene was first abolished by a frameshift mutation and an expression cassette containing the lacZ gene alone or together with the GFP gene was then inserted in place of the early protein 0 (*ep0*) gene of PRV. The reporter gene cassettes were positioned downstream from the PRV latency-associated promoter. The results revealed that the mutant PRV effectively infected human embryonic spinal cord neurons *ex vivo* and the grafted cells exhibited reporter gene expression for several weeks. [76].

Figure 14. *Human spinal cord graft cells infected with Prv-rrep0lacgfp virus before transplantation. The cells containing hu-NCAM (human cells) are red, GFP+ cells are green and the cells expressing both antigens are yellow. Confocal microscopic photographs (A, B and C) illustrate the same grafted spinal cord tissue after a survival time of 1 week in the dorsal horn of the host cord. In D, E and F human neurons can be seen 3 weeks after transplantation: the human neurons express GFP and possess well-defined processes. In G, host cells (arrows) are shown expressing GFP near the human graft 3 weeks after transplantation.*



PRV as a tool in cardiovascular research

To establish optimal surviving conditions several culture conditions were tested based on microscopic evaluation of changes in cellular morphology during the four days of culture (Figure 15). Cultured cells were used 1–3 days after isolation. During this period, visible small-scale changes in cell shape and cross-striation could be observed [5].

Figure 15. *This table shows the survival of noninfected and virally infected cells from day 0 to day 5. It can be seen that virally infected cells exhibit slightly better survival than noninfected cells, for which the reason remains to be ascertained.*

		Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Control	Average (%):	100	60,0	44,9	4,3	2,8	2,2
	Standard error:		6,0	8,4	0,5	0,3	0,3
Virus infected	Average (%):	100	85,5	69,2	12,8	3,1	1,3
	Standard error:		4,7	9,3	3,7	1,5	0,6

Features typical of acutely isolated (Day 0) cells were the rod shape with rectangular stepped ends and clear cross-striations. After 1 day (Day 1) in culture, the cells were still rod-shaped with clear cross-striations; however, the ends of the cells started to become slightly rounded in appearance. After 3 days (Day 3) in culture, cells remained rod-shaped and cross-striated, and the main change was that cell ends became progressively more rounded. Survival rates were found to be dependent on the isolation procedure, density of the attached myocytes, and the applied virus titer. Even after three days, the cell culture contained a substantial number of good quality cells both in the control and virus-infected groups. Surprisingly, a moderately but consistently higher cell survival rate was found in virus-infected groups as compared to non-infected groups. The infection

efficiency was found to be 100%, that is 24 hours post infection every surviving cells emitted fluorescent signals provided that high dose of virus was used for the infection [5].

DISCUSSION

Analysis of expression kinetics of regulator genes under CHX and PAA treatment

We employed a novel qRT^2-PCR technique with strand-specific primers for the reverse transcription reactions to determine the expression kinetics of specific PRV genes. Traditionally, herpes virus genes are grouped into kinetic classes on the basis of gene expression inhibitory effects of protein and DNA synthesis-blocking reagents. The IE genes should not be significantly inhibited by any of the above drugs, the E genes are inhibited only by DNA synthesis blockers, and the expression of the L genes is substantially inhibited by both protein and DNA synthesis blockade. The expression of the *ie180* gene is enhanced by CHX treatment. The explanation of this phenomenon lies in the fact that the IE180 protein normally inhibits its own expression a short time after the onset of virus infection, a feature which is absent in CHX-treated cells due to novel protein synthesis being blocked by CHX. The other PRV genes were inhibited to a significant extent by CHX at every examined time point. Consequently, ie180 is the only IE gene of the PRV. The ep0 and ul54 genes (IE genes in the HSV) were shown to be E genes by others [30 and 89], and this was confirmed also by our analyses [21]. The CHX analysis suggested that IE180 protein facilitates LAT activity, and inhibits AST. The PAA treatment resulted in a significantly elevated antisense transcript level at 3 of the 4 time points in antisense transcripts, which indicates the existence of another regulatory level besides IE180 protein action. We assume that transcription from one DNA strand negatively influences the expression of transcripts from the complementary DNA strand. The interaction can occur at the level of transcription (RNA polymerase moving in one direction along one of the DNA strands inhibits RNA polymerase moving in another direction) and/or translation by forming double-stranded RNAs by the sense and antisense transcripts. As an example, PAA has a negative effect on the transcription of *ie180* (the level of IE180 mRNA is reduced to a quarter) at 4 h pi, which results in a

lower rate of transcription from *ie180* (thereby facilitating the expression of AST); and a lower amount of inhibitory IE180 proteins, which also facilitates AST expression. Overall, the AST level increases 39.4-fold relative to the untreated conditions. Furthermore, genes with the same kinetic properties exhibit a distinctive distribution pattern along the PRV genome. Interestingly, *us1*, which is an IE gene in the HSV, appeared to be an L gene in most of our analyses; its expression was significantly inhibited by PAA; it afforded a low amount of mRNAs at 1 h pi, and a high rate of increase of transcript level at 4-6 h pi, all of these being L characteristics. Our explanation is that if the us1 gene has an important function early in the lytic cycle of the virus, it might fulfill this without de novo synthesis by being released upon infection from the tegument layer of the virus where it might be incorporated.

Dependence of expression properties of 37 PRV genes on the multiplicity of infection

In this study we infected PK-15 cells were with wild-type Ka strain of pseudorabies virus at MOIs of 0.1 and 10. Than we compared the gene expression pattern on the basis of infectious dose. We found that the mRNA levels of most examined PRV genes were higher in the cells infected with the high-MOI than in those infected with the low-MOI at early stages of infection. In the early stages of PRV infection, the amount of AST was very high; while the amount of AST and also its ratio to *ie180* mRNA were extremely low in the high-MOI infection. Moreover, *ie180* mRNA is expressed to a significantly higher extent in the low-MOI experiment despite the 10 times lower copy number of PRV DNA in an infected cell, which is especially important because IE180 is a DNA-binding protein. We think that this observation reveals an important regulatory mechanism of the herpes viruses, which is as follows: in a high-titer infection, the virus initiates a lytic infection in a cell, while in a low-titer infection, the virus has the choice of whether to establish a dormant state or enter a lytic cycle in a cell. The molecular mechanism of this phenomenon might be based on the interaction of *ie180* and AST genes at both the transcription and translation levels. (1) The ie180 protein might exert a negative effect on the synthesis of AST, such as in LAT in HSV [90] by binding the promoter of the antisense transcript. (2) Furthermore, the complementary transcripts might mutually influence each other's expression transcript by RNA-RNA interaction. In

a low-MOI infection, the two transcripts exhibit a complementary expression pattern, which indicates a competition between the two transcripts. In a high-MOI infection, however, the high initial amount of *ie180* gene product inhibits the expression of AST. The significance of this infection strategy could be that, in the case of a low-amount infection, the virus has no chance to invade the host cells; therefore, it is better to hide against the immune surveillance. Accordingly, similarly to AST, LAT is expressed in a significantly higher proportion to *ep0* mRNA in the low-MOI infection in the early stages of infection, which may also be important as concerns of the replication strategy of the virus. The expression of viral genes per DNA did not uniformly decreased in this analysis; some genes even became more active in the high-MOI infection, which indicates the selective effect of the reduced availability of the IE180 protein. We found in our analyses that AST and LAT are, at least partly, expressed independently from each other, which supports the existence of separate elements controlling the expressions of the two antisense transcripts. Indeed, AST was suggested to be controlled by an antisense promoter (ASP) localized in the outer regions of inverted repeats [91].

The effect of the MOI on the overall gene expression of HSV-1 has been investigated by Wagner and colleagues [92], who found that, following the infection of cultured cells by wild-type virus at MOIs ranging from 0.05 to 5 pfu/cell, the temporal profiles of transcript abundance were essentially the same. This is in sharp contrast with our results. We explain the differences by the low resolution of the microarray technique that Wagner et al. used for their analysis. An analysis of the global transcription of Rhesus monkey rhadinovirus, a γ-herpesvirus, has revealed differential gene expression at different MOIs [90], but these data cannot be compared because they related to later time points (12, 20, 44 68 and 93 h) than in our analysis.

Effect of vhs-negative mutant viruses on gene expression

Our results show that VHS protein is an important coordinator of global gene expression in PRV. In this study we have shown that at the early period of infection tegument VHS proteins affect the amount viral transcripts without bias toward any kinetic classes of viral transcripts. However, later on, *de novo* VHS protein appears to exert a differential negative effect on the level of early gene transcripts. We have also found that the

expression kinetics of PRV late transcripts are only slightly affected by the viral ribonuclease, while this effect on the level of E/L transcripts is intermediate. Our data suggest that a major function of the VHS protein is the assistance in the switch from the early to late phase of viral infection by selective inhibition of the early transcripts from 4 hour of infection. Theoretically, the effect of VHS protein on the PRV transcriptome might be indirect, that is, it can act through the regulation of mRNA level of one or more viral transactivators. Indeed, VHS protein exerts far the highest impact on EP0 transcripts among all of the examined PRV genes, suggesting that the *ep0* gene might be the major target of VHS effect, which does not exclude the possibility that VHS could affect other mRNAs in a direct manner, too. Altogether, we assume that VHS protein of PRV has an important role in the regulation of the PRV transcription cascade by its selective dampening effect of E transcripts, and to a lesser extent of E/L gene products at the late stages of viral infection.

PRVs as neuronal circuit tracers

With the deletion of *gE* and *gI* genes we could generate genetically modified PRVs, which are able to spread only in a retrograde manner. We engineered PRVs, the so called Ti-PRVs with both activity sensor and timer functions. Ti-PRVs could be used as internal clocks to determine a time window for functional studies [4].

These results arise from the collaboration of two research groups: the Department of Medical Biology, based in Hungary, and the Friederich Miescher Institute, based in Switzerland.

Dual-viral tracing

We have developed Ba-DupGreen and Ba-NeutRed fluorescent protein expressing viruses, which were used in dual viral transneuronal tracing method. This method was already successfully applied in numerous previous studies [93, 94 and 95]. In our experiments in which mixture of the two recombinant viruses was inoculated into the adrenal gland, the pattern and intensity of infection induced by the two virus strains were similar, that demonstrates that the main characteristics of the two recombinants, such as

invasiveness, rate of transport and replication do not differ from each other. Our results suggest that the dual transneuronal tracing with, we applied, reflects the supraspinal connections of the left adrenal gland and the left ovary [80].

This project was executed in cooperation between the Department of Medical Biology,Szeged, Hungary and the Research Laboratory of Neuromorphology and Neuroendocrinology, Budapest, Hungary.

PRVs in human embryonic cord neurons

We found that Prv-rrep0lacgfp is non-cytotoxic to the human embryonic cells as the PRV incubation did not increase the rate of cell death as compared with untreated tissues. Our observations suggest that this PRV strain is suitable for delivery of foreign genes into transplantable human cells.

These results due to the scientific cooperation with the Department of Ophtamology, Szeged, Hungary.

PRV as a tool in cardiovascular research

We showed that novel PRV-based vectors can transducer genes into cardiomyocytes, without causing cytotoxic effect on these cells. During our cell viability analysis we found, that electrophysiological measurements on these cells should be done within 4 days.

This project was performed in cooperation with the Department of Pharmacology and Pharmacotherapy, Szeged, Hungary.

Summary the results of

1. *Gene expression analysis*

We have examined the expression kinetics of the gene regulator genes under drug treatment.
We have observed the effect of viral load on PRV gene expression
We have shown that real-time RT-PCR is a useful method for the analyses of the effect of different gene-deleted mutant viruses on the PRV genome.

2. *PRV as neural circuit tracer*

We have developed genetically modified PRVs, which spread only in retrograde manner.
We have generated viruses for visualizing the connections of different brain regions.
We have generated viruses for the analysis of neural activity.
We combined the function of Timer and Activity sensor PRVs, to determine the time-period when functional measurements can be done.
We have engineered recombinant virus strains that are useful for the investigation of the central circuitry which is involved in the control of adrenal gland and ovary.

3. *PRV as gene-delivery vector*

We have developed PRVs which have no cytotoxic effect on the infected cells and are suitable for gene delivery into transplantable human cells and cardiomyocytes.

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