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Deletion of the virion host shut-off gene of pseudorabies virus results in selective upregulation of the expression of early viral genes in the late stage of infection

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ABSTRACT

A real-time RT-PCR technique was applied to evaluate the impact of deletion of the virion host shut-off (VHS) gene on the kinetics of pseudorabies virus gene expression. Selective suppression of early gene transcripts by the viral ribonuclease occurs after 4 h of infection; while VHS protein appears to act non-selectively on the transcripts belonging in different kinetic classes in the first 2 h of infection. VHS protein disrupts the close correlation between the transcription kinetics of the immediate-early 180 protein and the other pseudorabies virus transcripts. The typical pattern of early gene expression was found to be altered in the VHS gene-deleted virus in that the production rates of their transcripts did not decline from 4 h post-infection. This observation led us to put forward the hypothesis that the VHS protein may play a pivotal role in the switch from the early to the late stage of infection.

1. Introduction

Pseudorabies virus (PRV), a neurotropic herpesvirus, is an important pathogen of swine, causing Aujeszky's disease [1]. PRV is a model organism widely used to study the molecular pathogenesis of herpesviruses [2,3], and it is also applied as a transneuronal tract tracing tool [4–6]. PRV has 70 protein coding genes, which belong in four major temporal classes: the immediate-early (IE), early (E), early-late (E/L) and late (L) classes. The immediate early 180 gene (*ie180*), the only IE gene of PRV (homologous to the HSV ICP4 gene), plays an essential role in the transactivation of viral gene expression [7]. The early protein 0 gene (*ep0*; ortholog of the HSV ICP0 gene) is another important transcription factor of PRV, whose function in the regulation of the overall viral transcription is largely unknown [8]. The virion host shutoff (VHS) protein, encoded by the PRV *ul41* gene, acts as an mRNA-specific endoribonuclease [9]. *ul41* is expressed as an L gene, and is nonessential, since a *vhs*-knockout virus retains its capability to grow both in cultured cells and *in vivo*[10]. However, deletion of the VHS function results in a 5- to 10-fold reduction in virus yield in a cell culture [11,12] and in a significant loss of virulence [13], which suggests that VHS plays an important role in the viral infection [14,15]. The VHS protein causes the degradation of both viral and cellular mRNAs [16], but is unable to degrade the tRNAs and rRNAs of the host cell [17]. Further functions associated with VHS activity are to ensure ribonucleotides for the viral transcription machinery, and to preclude host responses to infection by the elimination of mRNAs of specific cellular genes, such as TNF-receptor 1 gene [11]. The VHS proteins are expressed in the late stage of viral infection, and are incorporated into the tegument of the virion [11,18,19]. The enzymatic activity of tegument-derived VHS ceases in the early phase of viral infection [20]. It has been reported that the nuclease activity of the *de novo* VHS protein is significantly reduced, although it is accumulated in a large amount. This phenomenon is explained by the inhibitory effect of viral factors VP16 and VP22 on the RNase activity of VHS molecules [12,21–23]; or alternatively, by enabling the translation of viral mRNAs by these factors [20]. Thus, according to the currently accepted scenario, VHS released from the tegument of the penetrating virus degrades the viral mRNAs, and especially those with early kinetics, while the *de novo* synthesized VHS molecules are largely inactive, possibly because they are packaged into the newly assembled virions. In this study we assessed the effects of the deletion of VHS function on the expression kinetics of 38 PRV genes by using a knockout technique for elimination of the VHS gene and a reverse-transcription real-time PCR (RT^2-PCR) assay, a high-resolution genomics technique, for analysis of the PRV transcriptome. Our data suggest a different mechanism of VHS action than those of earlier studies, which mainly used co-transfection reporter gene systems. The limitations of these approaches are the low resolution of obtained data, the absence of viral proteins that might modify the RNase activity of the VHS molecules, and the low number of genes that can be simultaneously analyzed. In accordance with the logic of knock-out studies, we assume a reverse effect of the VHS protein in the wild-type virus, which was observed in the *vhs*-null mutant virus, emphasizing that this effect is not necessarily direct. Indeed, we hypothesize that the VHS protein may act on the PRV transcriptome through the regulation of EP0 gene expression.

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Furthermore, albeit the *vhs* gene was knocked out, the effect of VHS protein is naturally missing in the mutant virus.

2. Results and discussion

2.1. Expression of VHS mRNAs in wild-type PRV

Our RT^2-PCR data reveal a low-level expression of the VHS transcripts in the first 2 h of infection in the wild-type (*wt*) PRV. The transcript level starts to rise significantly from 2 to 4 h and reaches its maximum at 24 h with local maxima at 6 and 12 h post infection (pi; Fig. 1), which is typical expression kinetics for an L PRV gene. On the basis of these and other data [15], it is reasonable to assume that tegument VHS proteins play a role in the very early stage of infection, while *de novo* VHS molecules take over the ribonuclease function between 2 and 4 hpi.

2.2. The expression kinetics of early genes is basically altered in the vhsΔ virus

VHS molecules modify the transcript levels of each individual gene in a varying manner (Supplemental Figure S1), but we observed important regularities of these effects typical of the different kinetics classes of genes (see below). The data demonstrated that all of the examined PRV transcripts are expressed at a lower level from the *vhsΔ* genome than from that of the wt ($R_r = R_{\Delta vhs}/R_{wt} < 1$) at 1 hpi (Fig. 2). Our explanation for this phenomenon is as follows. Although viral mRNAs are also the targets of VHS protein [24,25], the preferential degradation of cellular mRNAs [15] by the viral RNAse at this stage of infection might compensate this effect in two different ways: by elimination of the competition between the host and the virus for the translation apparatus of the cell in favor of the virus, and the production of new ribonucleotides serving as raw materials for the newly synthesized viral transcripts. We have found no bias of the VHS effect toward any kinetic class of PRV genes at this time of infection. At 2 hpi, VHS proteins exert an inhibitory effect on the accumulation of PRV transcripts in all three kinetic classes (E, E/L and L; Fig. 2) with a few exceptions. Thus, VHS proteins appear to switch their inhibitory effect toward viral mRNAs, but without distinction between the kinetic classes of viral transcripts within 1 to 2 hpi. There is an overall fall in the VHS effect in the period 2 to 4 hpi (Fig. 3, Table 1) ($\overline{R_r} = \overline{R}_{\Delta vhs} / \overline{R}_{wt}$). The gene expression curves show that the average transcript levels of the E/L and L genes in the *vhs* null mutant become practically the same as in the *wt* virus at 4 hpi. This is in contrast with the E transcripts, whose average levels remain higher in the *vhs* mutant strain than in the *wt* virus, but their levels are also somewhat lower at 4 h than at 2 hpi in the mutant PRV. We explain this phenomenon by the dampening level of tegument VHS proteins, which uniformly act on the different kinetic classes of viral genes and the rising level of newly synthesized 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 the *vhs*-knockout than in the *wt* virus from 4 to 24 hpi, which is most marked at 8 and 12 hpi (Supplemental Figure S1; Figs. 2 and 3). The average late gene expressions were not significantly changed in the mutant virus as compared to the *wt* virus at 4 hpi. There are some L genes whose transcript levels are even lower in the *vhsΔ* background than that in of the wt at many time points of infection, e.g. the *ul1*, *ul5*, *ul10*, *ul19*, *ul44*, *ul48* and *us1* genes (Supplemental Figure S1). The average levels of E/L transcripts always fall between the E and L levels in the interval 6 h to 24 hpi. No selective effect of VHS activity is observed in the E/L and L kinetic classes of PRV genes at 4 and 6 hpi. In fact, the average effects of the VHS protein on the E/L and L transcripts are similarly low in the first 6 hpi, while this is not the case for the E genes of the virus (Fig. 3). The $\overline{R_r}$ value of the E genes rapidly increases within the period 4 h to 12 h, but drops sharply by 18 hpi; however, the amounts of these transcripts remain higher in the *vhs*-deleted virus than in the *wt* virus even at this time (18 h). The $\overline{R_r}$ value of the E/L transcripts becomes larger than 1 at each time point between 6 and 18 h, peaking at 8 hpi at a medium level relative to the E mRNAs. Our data indicate that there is no selective effect of the VHS protein on the E/L transcripts at 24 hpi. The transcripts of the L genes are only slightly affected by the VHS proteins within the interval 6 h to 24 hpi, with a peak of $R_r = 1.56$ at 12 hpi (Table 1). Fig. 3 shows that the VHS protein exerts its strongest effect on the E and E/L genes between 8 and 12 hpi. Together, VHS protein exert a high inhibitory effect on the transcript levels of E genes ($r = 0.582$), an intermediate effect on E/L ($r = 0.894$) genes and hardly any effect on L gene expression ($r = 0.960$) (Table 2A).

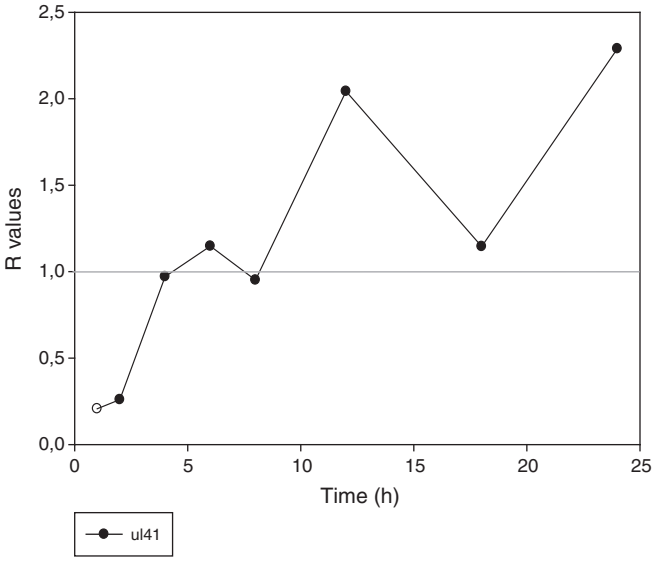

Fig. 1. Expression kinetics of the *ul41* (*vhs*) gene in the *wt* virus. The plot of relative expression ratios (*R* values) shows that the level of the *vhs* transcript is very low in the first 2 hpi; there are local maxima at 6 and 12 h; the overall maximum is reached at 24 hpi.

Comparison of the expression curves of the *R* values of the *ie180* gene with the average for other PRV genes in the two genetic backgrounds gives the same result as above: the kinetics of *ie180* mRNA and the averages of the remaining viral transcripts were unrelated in the *wt* strain, but were very similar in *vhsΔ*, and this held true for the averages of each kinetic class of genes (Fig. 4A, C and Supplemental Figure S2). We obtained the same results if we used the R_Δ values for the comparison as above (Fig. 4B, D and Supplemental Figure S2). What can the reason be for the differential effect of the VHS protein on the transcripts belonging in various kinetic classes? Fig. 4A illustrates a typical dynamics of gene expression curves in the *wt* background: the E genes are expressed at a high level at the early stage of infection, followed by a decline in the rate of mRNA production from 4 hpi, while the amounts of the L gene products increase rapidly within the period 6 to 12 hpi. This expression pattern is broken in the *vhsΔ* virus, which is most markedly manifested in the non-declining rate of E gene expression in the period 4 to 12 hpi. Examination of the individual E genes reveals that their maximal expressions are shifted from 4 and 6 h to 8 and 12 h in most cases (Supplemental Figure S1). Furthermore, while the average *R* values of the L genes in the *wt* virus are higher than those of the E genes (E = 1.210; E/L = 1.045; L = 1,297), in *vhsΔ* the situation is basically changed in favor of the E genes (E = 2,653; E/L = 1,582; L = 1,516) (Fig. 4A). Overall, the absence of the VHS protein renders the kinetics of the E gene expressions similar to those of the L genes. These results

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	***ul52***	0,505	2,071	1,957	2,111	4,142	2,585	1,121	0,541
E	***ul54***	0,294	1,893	0,917	1,774	3,986	3,566	1,836	2,633
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
	AST	0,288	1,298	0,409	0,776	1,087	1,229	1,176	0,997
	LAT	0,376	2,553	0,415	0,934	1,176	2,495	0,736	0,618

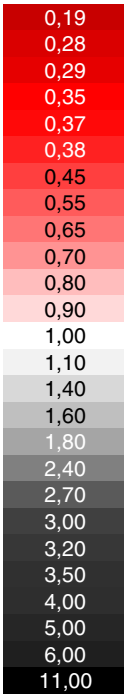

Fig. 2. Heatmap-like visualization of transcript ratios in the two viruses ($R_r = R_{vhs\Delta}/R_{wt}$) at the examined time points. The genes are arranged according to their kinetic classes. This figure reveals a dramatic elevation in the expression levels of the E and E/L genes at 8 and 12 hpi in the mutant virus. The color code is as follows: dark-black = highest R_r values; dark-red = lowest R_r values; white = values close to 1.

suggest a critical role of the VHS protein in the transition from the early to late phase of PRV gene expression.

2.3. The VHS activity disrupts the synchronicity of viral gene expression

Comparison of the R_Δ values of the PRV transcripts of the mutant and wt virus reveals a peculiar pattern of gene expression kinetics (Fig. 5). In the *wt* virus, the transcript levels of the individual genes change in an irregular manner with time with respect to whether the amount of the transcript increase, decrease or stagnate, except within the infection period 2 to 4 h, where none of the viral transcripts decrease (i.e. they either increase or stagnate). However, in the *vhs*-deleted virus the transcript levels do not decrease (i.e. they only increase or stagnate) from 1 h until 8 h in every gene, with the exception of *us1* within the period 6 to 8 hpi. Furthermore, in the *vhs* null mutant the transcript levels uniformly drop in the infection period 12 to 18 h, which is not the case in the *wt* virus. This phenomenon can only be partly explained by the selective shut-off of E transcripts, because the expression of some L genes is also dampened in the *vhsΔ* strain. The general decrease in gene transcript levels in the period 12 h to 18 hpi might be explained by the drop in level of the EP0 transcripts in the *vhs* null mutant (for an explanation, see below).

2.4. The expression kinetics of ie180 transcripts correlates with the average kinetics of other viral transcripts in the absence of VHS activity

Pairwise comparisons of the *R* values of the *ie180* gene with the average *R* values of other PRV genes at different time points by means of Pearson correlation analysis revealed a high correlation for the *vhs* null mutant ($r = 0.893$), while only a very low correlation was found for the *wt* virus ($r = 0.330$; Table 2B). There was no significant bias in the correlation toward any kinetic class of PRV genes (Table 2A). The expression kinetics of the *ie180* gene was also affected by deletion of

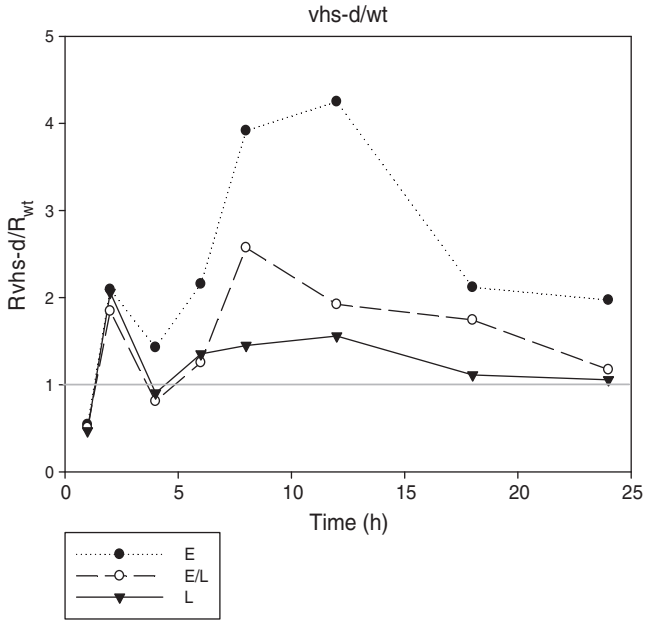

Fig. 3. Plots of the average R_r values of the E, E/L and L transcripts ($\overline{R_r} = \overline{R}_{\Delta vhs} / \overline{R}_{wt}$) versus time. These plots show that there is an overall fall in the effect of the VHS protein (decrease in $\overline{R_r}$ values) between 2 and 4 hpi. Further, the transcription and/or stability of the E mRNAs are strongly affected by the VHS molecules, while this effect is much weaker for the L transcripts and intermediate for the E/L transcripts.

the *vhs* gene (Supplemental Figure S1; Table 2C). Comparison of the $R_r = R_{\Delta vhs}/R_{wt}$ values of the *ie180* transcripts with those of other PRV genes revealed a differential correlation in the transcription kinetics between the *ie180* and the other PRV genes (Table 2D; Fig. 6). The increase in the *ie180* transcript level in the *vhs* null mutant relative to the *wt* virus (R_r value) is strongly correlated with that in the E gene transcripts ($r = 0.952$), and moderately correlated with that in the E/L transcripts ($r = 0.803$), while there is only a very week correlation between *ie180* and the L transcripts ($r = 0.385$). We interpret these results as follows. The IE180 protein is known to coordinate the expressions of the PRV genes by promoting them. Our results suggest that the effect of the IE180 protein on the overall viral gene expression is modified by the VHS protein in the *wt* virus, which explains the lack of a correlation between *ie180* and other transcripts in that virus. The question as to whether this modifying effect is exerted by the VHS protein in a direct or an indirect manner is discussed below.

2.5. VHS may exert its effect through regulation of the level of ep0 transcripts

Our data show that the VHS protein has by far the greatest impact on the transcript level of the early protein 0 gene (*ep0*) among all of the examined 38 PRV transcripts (Fig. 7; Table 2E). In the first hour pi, similarly to other genes, the VHS protein exerts a strong stimulatory effect on the transcription of the *ep0* gene. Later, the VHS protein acts to lower the copy number of EP0 mRNAs, which culminates at 12 hpi, where the level of the *ep0* transcripts is more than 11 times higher in *vhs* null mutant than in the *wt* virus. Furthermore, the *ep0* transcription kinetics is highly correlated with that of *ie180* in the *vhs* null mutant virus ($r = 0.858$), which is not the case at all in the *wt* virus, where there is no correlation in the expression kinetics between

Table 1
Average R_r values of PRV transcripts belonging in different kinetic classes. This Table shows the $\overline{R_r}(=\overline{R}_{\Delta vhs} / \overline{R}_{wt})$ values of E, E/L and L transcripts at the examined time points (1, 2, 4, 6, 8, 12, 18 and 24 hpi).

Kinetic class	1 h	2 h	4 h	6 h	8 h	12 h	18 h	24 h
E	0.543	2.093	1.429	2.157	3.917	4.250	2.119	1.972
E/L	0.509	1.846	0.811	1.256	2.573	1.923	1.743	1.174
L	0.471	2.060	0.906	1.355	1.452	1.561	1.113	1.057

Table 2
Pearson's correlation coefficients of PRV genes. (A) Pearson's correlation analysis was performed to examine the relationship between genes belonging in the same kinetic group in different genetic backgrounds. The correlation is lowest between the E genes, and highest between the L genes. (B) Pearson's correlation analysis was performed, using the R values of the *ie180* transcript and the average *R* values of the other PRV mRNAs. Data show high correlations between the *ie180* and the other transcripts in the *vhs*-knockout background, while there is hardly any correlation in the *wt* virus. (C) This Table shows the Pearson's correlation coefficients between *ie180 mRNAs* in the *vhs*-mutant and the *wt* backgrounds. (D) Pearson's correlation coefficients were measured to examine the relationship between the R_r value of *ie180* and the average R_r values of the other viral transcripts. It is seen that the *ie180* transcripts are very highly correlated with the E transcripts, while there is a lower level of correlation with the E/L transcripts, and hardly any correlation between the *ie180* and L transcripts. (E) Pearson's correlation between the *ep0* transcripts in the *vhs* mutant and the *wt* backgrounds. It can be seen that there is no correlation between the *ep0* transcription patterns in the two different genetic backgrounds. (F) Pearson's correlation analysis was used to determine the relationship between the *ie180* and *ep0* transcripts in the two genetic backgrounds of the virus. This Table shows a high correlation between the transcript levels of the two regulatory genes in the *vhs*Δ background, but they are uncorrelated in *wt* background. (G) This Table lists the Pearson's correlation coefficients between *ep0* and the other PRV transcripts. There is a high positive correlation between the *ep0* and the other PRV transcripts in the *vhs* null mutant virus, and a negative correlation between them in the *wt* background.

A.

	r Values
$\overline{R}_{vhs\Delta \mathbf{E\ genes}}$ vs.$\overline{R}_{wt\mathbf{E\ genes}}$	0.582
$\overline{R}_{vhs\Delta \mathbf{E/L\ genes}}$ vs.$\overline{R}_{wt\mathbf{E/L\ genes}}$	0.894
$\overline{R}_{vhs\Delta \mathbf{L\ genes}}$ vs.$\overline{R}_{wt\mathbf{L\ genes}}$	0.960

B.

	r (wt)	*r (vhsΔ)*
$\boldsymbol{R_{ie180}}$ vs.$\overline{R}_{\mathbf{other\ genes}}$	0.330	0.893
$\boldsymbol{R_{ie180}}$ vs.$\overline{R}_{\mathbf{E\ genes}}$	0.237	0.875
$\boldsymbol{R_{ie180}}$ vs.$\overline{R}_{\mathbf{E/L\ genes}}$	0.411	0.882
$\boldsymbol{R_{ie180}}$ vs.$\overline{R}_{\mathbf{L\ genes}}$	0.321	0.900

C

	r
$\overline{R}_{vhs\Delta \boldsymbol{ie180}}$ vs.$\overline{R}_{wt\ \boldsymbol{ie180}}$	0.574

D.

	r (vhsΔ/wt)
$\boldsymbol{R_{r\text{-}ie180}}$ vs.$\overline{R_r}_{\mathbf{other\ genes}}$	0.811
$\boldsymbol{R_{r\text{-}ie180}}$ vs.$\overline{R_r}_{\mathbf{\text{-}E\ genes}}$	0.952
$\boldsymbol{R_{r\text{-}ie180}}$ vs.$\overline{R_r}_{\mathbf{\text{-}E/L\ genes}}$	0.803
$\boldsymbol{R_{r\text{-}ie180}}$ vs.$\overline{R_r}_{\mathbf{\text{-}L\ genes}}$	0.385

E

	r
$\overline{R}_{vhs\Delta \boldsymbol{ep0}}$ vs.-$\overline{R}_{wt\ \boldsymbol{ep0}}$	−0.188

F

	r (wt)	*r (vhsΔ)*
$\boldsymbol{R_{ep0}}$ vs.$\boldsymbol{R_{ie180}}$	0.210	0.858

G.

	r (wt)	*r (wt)*
$\boldsymbol{R_{ep0}}$ vs. $\overline{R}_{\mathbf{other\ genes}}$	−0.571	0.868
$\boldsymbol{R_{ep0}}$ vs. $\overline{R}_{\mathbf{E\ genes}}$	−0.566	0.882
$\boldsymbol{R_{ep0}}$ vs. $\overline{R}_{\mathbf{E/L\ genes}}$	−0.525	0.819
$\boldsymbol{R_{ep0}}$ vs. $\overline{R}_{\mathbf{L\ genes}}$	−0.567	0.868

the two transcription factor genes ($r = 0.210$) (Fig. 8A, Supplemental Figure S2 and Table 2F). In the *vhsΔ* strain, there is a high correlation between the *ep0* transcripts and the other PRV gene products ($r = 0.868$ on average), while there is an inverse correlation between the *ep0* mRNA and the other PRV transcripts ($r = -0.571$) in the *wt* virus (Fig. 8B, Supplemental Figure S2 and Table 2G). It cannot be excluded that VHS exerts its effect on the level of the PRV transcripts through regulation of the *ep0* mRNAs. According to this scenario, the VHS proteins act to lower the EP0 protein level by degrading the *ep0* mRNAs, which in turn results in a selective lowering of the E transcripts. Indeed, the *ep0* mRNA reaches its maximum at 4 hpi in the *wt* virus, while it peaks at a very high level at 12 h in the *vhsΔ* virus (Fig. 7). This delay in the expression maximum in the *ep0* gene may account for the same delay in the maximal expression of the E genes. Furthermore, the drop in the *ep0* transcript level from 4 hpi may explain the decline in the rate of production of the E gene transcripts in the *wt* virus. Furthermore, the similar transcription kinetics of the *ie180* and *ep0* transcripts in the *vhsΔ* virus may account for both the synchronized viral gene expression and the high correlation of the transcript levels of the PRV genes within the *ie180* transcript level in the *vhs* mutant virus. For an explanation, in contrast with the *vhsΔ* strain, in the *wt* virus the *ep0* gene is expressed with different kinetics

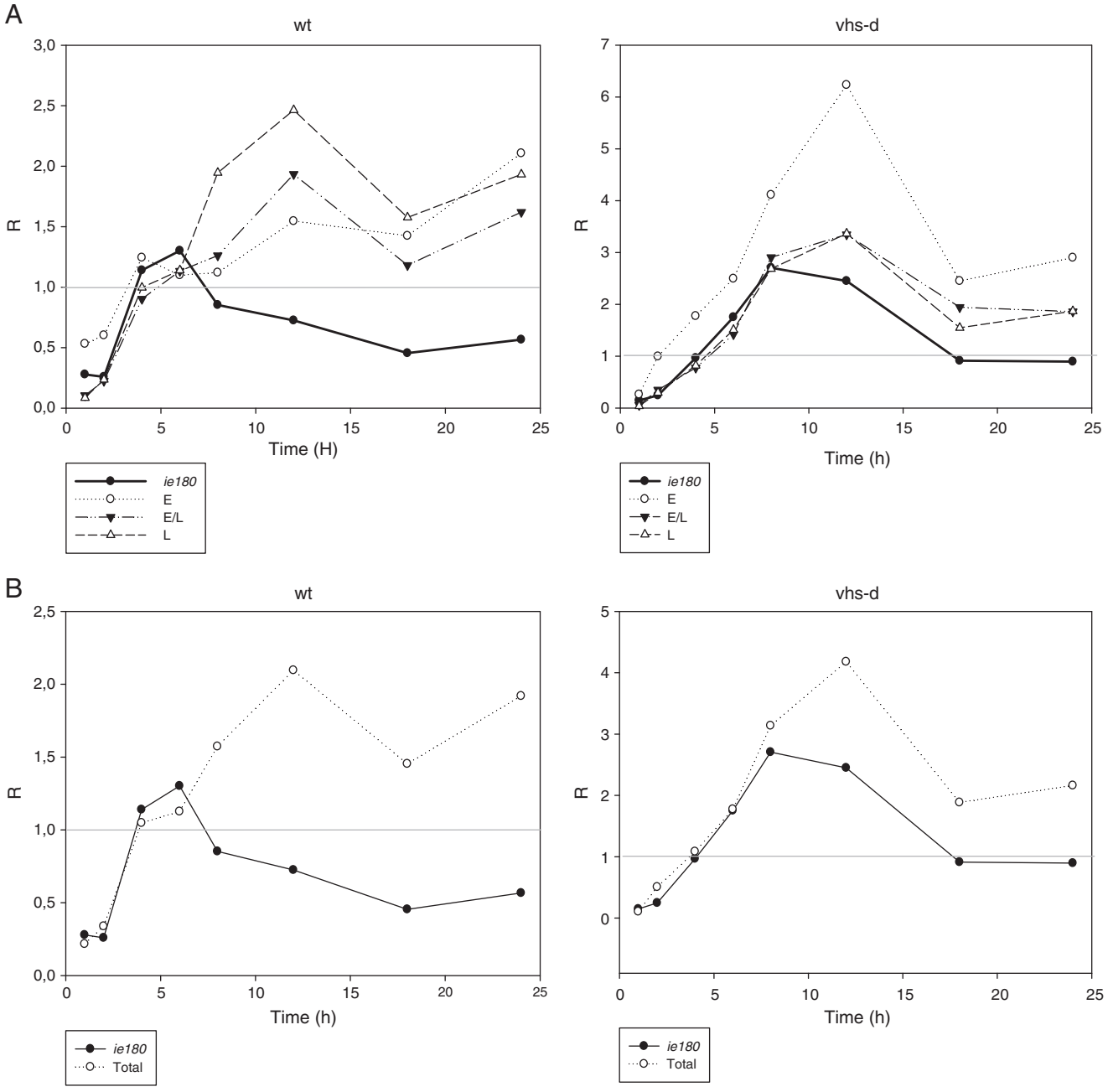

Fig. 4. The VHS protein acts to alter the pattern of gene expression of early PRV genes (A) The average expression kinetics of E genes (illustrated by using $\overline{R}$ values) exhibits a typical pattern in the *wt* virus: their expression rates decrease from 4 hpi and in most E genes have low values in the late stages of infection. In contrast, in the *vhsΔ* virus, the transcript levels of E genes do not drop from 4 hpi, their average amounts exceed significantly the levels of L transcripts in every time point of infection in the mutant virus. (B) The running curve of the average *R* values of total PRV transcripts is similar to that of ie180 transcripts' in vhsΔ virus, which is not the case in the *wt* virus. The above statements are illustrated by using the average R_Δ values ($\overline{R}_\Delta$) of E, E/L and L transcripts (C); as well as the average of the total PRV transcripts (D).

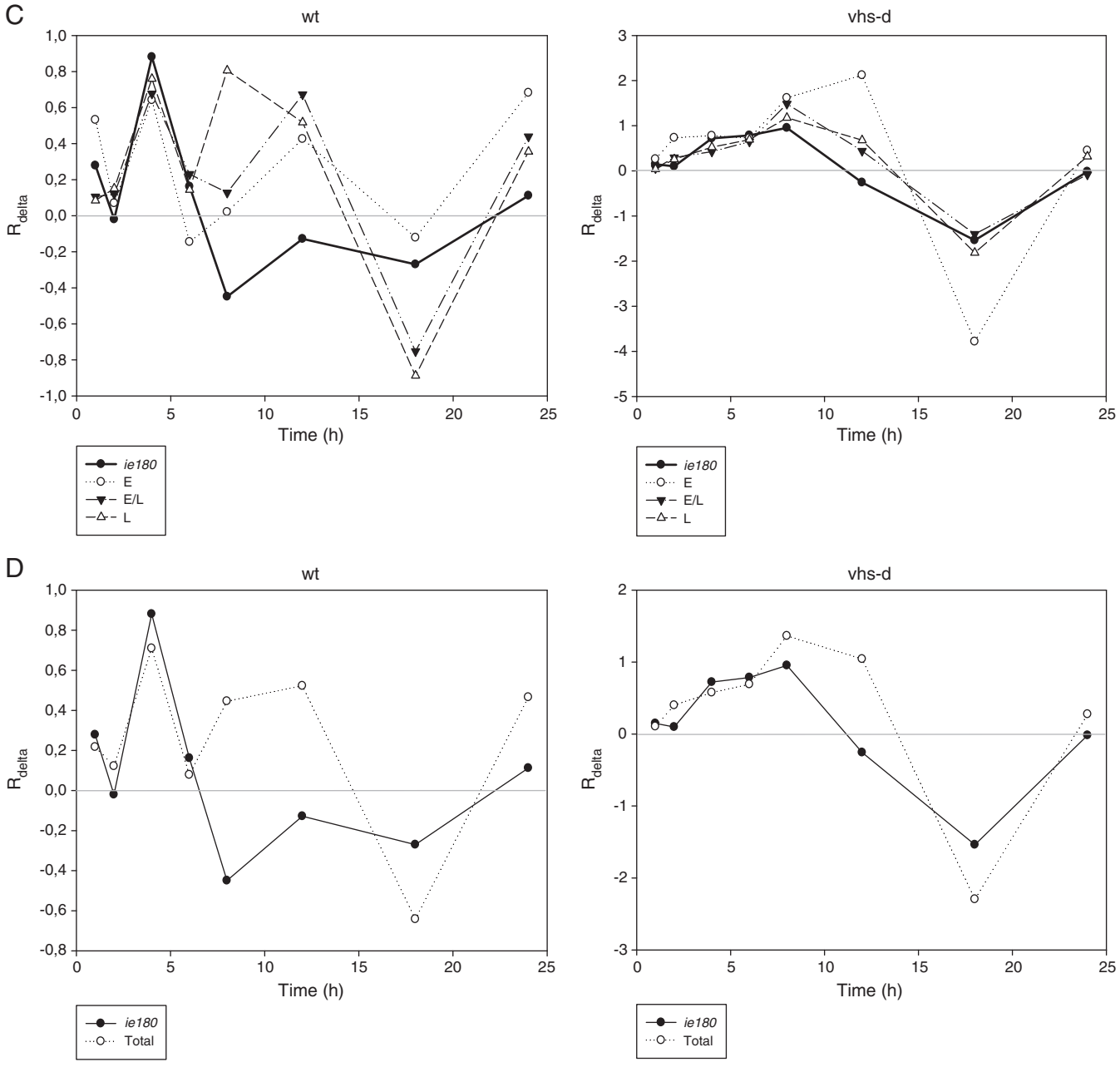

Fig. 4 (*continued*).

from that for the *ie180* gene, which may disrupt the correlation between the amount of the IE180 protein and the other PRV gene products. We intend to explore this possibility by deleting the *ep0* gene of PRV and examining the effects of the mutation on the global gene expression of PRV in a following study.

3. Conclusions

Our results demonstrate that the VHS protein is an important coordinator of global gene expression in PRV. This study has revealed that in the early period of infection tegument VHS proteins affect the amount of viral transcripts without bias toward any kinetic class of viral transcripts. Later, however *de novo* VHS protein exerts a differential negative effect on the level of early gene transcripts. We also found that the kinetics of expression of the PRV late transcripts is only slightly affected by the viral ribonuclease, while the effect on the level of E/L transcripts is intermediate. Furthermore, the VHS protein destroys the strong correlation in the expression between *ie180* and the other PRV genes. Our data suggest that a major function of the VHS protein is to assist in the switch from the early to late phase of viral infection by selective inhibition of the early transcripts from 4 hpi. Theoretically, the effect of the VHS protein on the PRV transcriptome may be indirect, i.e., it can act through regulation of the mRNA level of one or more viral transactivators. Indeed, the VHS protein exerts by far the greatest effect on *ep0* transcripts among all of the examined PRV genes, suggesting that the *ep0* gene may be the major target of VHS action, which does not exclude the possibility that VHS could affect other mRNAs in a direct manner too (Fig. 9). Overall, we assume that the VHS protein of PRV has an important role in regulation of the PRV transcription cascade through its selective dampening effect on the early transcripts, and to a lesser extent on the E/L gene products in the late stages of viral infection.

A

Gene	2h-1h	4h-2h	6h-4h	8h-6h	12h-8h	18h-12h	24h-18h
ie180	-0.020	0.881	0.162	-0.449	-0.128	-0.270	0.112
ep0	0.213	0.716	-1.273	-0.134	0.087	-0.441	0.083
ul9	0.064	0.378	0.335	0.392	0.294	1.299	-0.702
ul21	0.054	0.744	0.222	1.046	-0.290	-0.280	0.118
ul23	0.267	1.064	-0.520	-0.161	0.072	-0.170	0.199
ul29	0.330	0.548	-0.209	-0.410	0.148	-0.192	0.023
ul30	-0.397	0.255	0.326	-0.200	0.390	0.131	0.727
ul50	-0.178	0.995	-0.194	-0.388	1.112	-1.022	0.885
ul52	0.055	0.471	0.020	0.386	2.035	0.158	5.055
us3	0.344	0.604	-0.272	0.034	-0.031	-0.426	0.193
ul14	0.108	0.731	0.045	-0.010	1.139	0.326	0.085
ul15	0.146	0.462	0.350	-0.131	-0.033	-0.258	0.160
ul20	0.137	0.558	0.451	-0.349	0.761	-1.043	0.286
ul43	0.123	0.946	0.071	0.256	-0.101	-0.389	1.105
ul53	0.237	0.831	-0.193	-0.168	2.404	-1.784	0.727
us6	0.015	0.419	0.543	0.783	0.544	-1.189	-0.089
us9	0.070	0.795	0.348	0.513	-0.005	-0.931	0.800
ul1	0.063	0.852	0.167	0.433	-0.064	-0.587	0.037
ul5	0.977	0.972	-1.626	3.033	-2.805	1.327	-0.938
ul6	0.765	0.039	0.349	0.497	-0.398	-0.420	0.147
ul10	0.039	0.631	0.312	1.449	1.233	-1.596	0.464
ul17	0.064	0.968	0.005	0.933	0.739	-0.806	0.777
ul19	0.018	0.754	0.566	1.450	-0.813	-0.204	-0.053
ul22	0.074	0.267	0.762	-0.571	0.269	-0.538	0.223
ul24	-0.001	0.919	0.082	0.167	0.201	0.013	0.946
ul32	0.095	1.936	-0.545	1.534	0.317	-1.463	0.328
ul33	0.017	0.478	0.559	0.149	0.369	-0.266	0.801
ul36	-0.142	0.445	0.402	0.563	0.789	-0.697	-0.013
ul37	0.031	0.685	0.300	0.393	0.131	-0.334	0.179
ul40	0.031	1.322	-0.072	0.246	0.498	-1.696	0.236
ul42	0.103	0.922	-0.057	0.466	0.211	-0.631	-0.280
ul44	0.014	0.528	0.516	0.762	2.105	-3.246	0.671
ul48	0.013	0.710	0.396	1.023	2.297	-1.346	2.303
ul49,5	0.018	0.573	0.404	0.594	1.544	-1.650	0.977
ul54	-0.051	0.646	0.113	-0.356	0.444	-0.270	0.243
ul51	0.593	0.741	-0.365	2.138	3.755	-3.143	-0.116
us1	-0.014	1.256	0.357	-0.510	-0.547	-0.391	-0.053
AST	-0.001	0.094	0.020	0.104	0.168	-0.132	0.019
LAT	0.053	1.171	-0.257	1.178	0.527	-0.370	0.438

B

Gene	2h-1h	4h-2h	6h-4h	8h-6h	12h-8h	18h-12h	24h-18h
ie180	0.059	0.761	0.785	0.953	-0.256	-1.537	-0.017
ep0	1.681	2.737	2.531	0.477	7.696	-12.137	0.078
ul9	0.533	0.198	0.391	1.164	1.221	-0.600	-0.466
ul21	0.353	1.145	1.068	3.009	4.754	-7.521	1.189
ul23	0.442	0.693	0.756	2.170	-0.573	-2.297	1.525
ul29	1.047	0.427	0.175	1.761	-0.964	-1.843	-0.067
ul30	0.793	0.019	0.353	0.875	1.777	-2.313	0.259
ul50	0.977	0.219	0.713	1.430	1.173	-1.700	0.352
ul52	1.002	0.850	0.210	3.858	2.926	-4.995	0.589
us3	0.142	1.148	0.057	0.606	1.881	-2.031	-0.245
ul14	0.435	0.201	0.846	1.553	1.757	-1.536	-0.238
ul15	0.189	0.214	0.464	0.460	1.075	-1.729	0.279
ul20	0.281	0.757	-0.020	2.216	-0.862	-0.880	-0.161
ul43	0.323	0.699	1.243	2.619	-0.582	-1.527	-0.393
ul53	0.316	0.886	1.011	1.649	0.397	-1.694	-0.541
us6	0.162	-0.036	0.397	0.409	0.633	-0.776	0.163
us9	0.357	0.248	0.584	1.483	0.661	-1.675	0.307
ul1	0.243	0.235	0.287	0.611	0.542	-1.227	0.363
ul5	0.537	-0.004	0.695	3.225	-1.930	-1.436	0.565
ul6	0.369	0.203	0.780	0.729	0.086	-0.717	-0.201
ul10	0.109	0.562	0.591	1.222	0.021	-0.690	-0.348
ul17	0.067	1.295	0.243	1.193	1.982	-2.140	0.517
ul19	0.148	0.415	0.846	1.850	-0.096	-2.616	-0.105
ul22	0.191	0.513	0.261	0.717	-0.440	-0.610	0.384
ul24	0.117	0.671	0.899	1.119	0.043	-1.495	1.408
ul32	0.367	1.607	0.488	1.183	0.548	-2.383	0.452
ul33	0.306	0.475	0.487	1.254	-0.178	-0.481	0.643
ul36	0.403	0.394	0.875	0.857	-0.213	-1.017	-0.061
ul37	0.161	0.540	0.953	2.075	-0.136	-2.781	0.125
ul40	0.358	0.379	0.085	0.852	0.958	-1.754	0.178
ul42	0.166	0.413	0.279	0.275	0.939	-1.178	0.299
ul44	0.024	0.192	0.182	0.634	0.719	-0.972	0.100
ul48	0.121	0.365	0.295	1.533	2.059	-1.691	0.778
ul49,5	0.135	0.325	0.325	0.344	3.835	-2.921	-0.152
ul54	0.381	0.351	0.967	0.810	1.309	-2.391	1.298
ul51	1.085	0.449	3.416	2.858	3.801	-7.337	1.278
us1	0.067	0.779	0.522	-0.490	0.629	-0.992	-0.288
AST	0.007	0.034	0.053	0.150	0.238	-0.176	-0.027
LAT	0.345	0.166	0.455	1.645	4.285	-4.985	-0.052

Fig. 5. Two-color heatmap representation of the R_{Δ} values of the PRV transcripts in the *wt* (A) and the mutant virus (B) background. It can be seen that the directions of the changes in the gene expressions are more similar for the genes in the *vhs* gene-deleted strain than for those in the *wt* virus, which suggests a role of the VHS protein in disrupting a gene expression synchronization mechanism. The color code is as follows: black = an increase or stagnation between two time-points; white = a decrease between two time-points.

4. Materials and methods

4.1. Cells and viruses

Immortalized porcine kidney-15 (PK-15) cells were used for the propagation of pseudorabies virus (PRV). Cells were cultivated in Dulbecco's Modified Eagle Medium (Sigma-Aldrich) supplemented with 5% fetal bovine serum (Gibco) and 80 μg of gentamicin (Invitrogen™) per ml, at 37 °C in an atmosphere of 5% CO_2. The wild-type (*wt*) strain Kaplan of PRV (wt PRV) was used as a parental virus to generate *vhs*-knockout PRV.

4.2. Generation and characterization of vhs-null mutant pseudorabies virus

The *vhs*-knockout PRV was generated by homologous recombination as described earlier [10]. Briefly, the *Xho*I DNA fragment containing the entire *ul41* gene was subcloned to the *Sal*I site of the

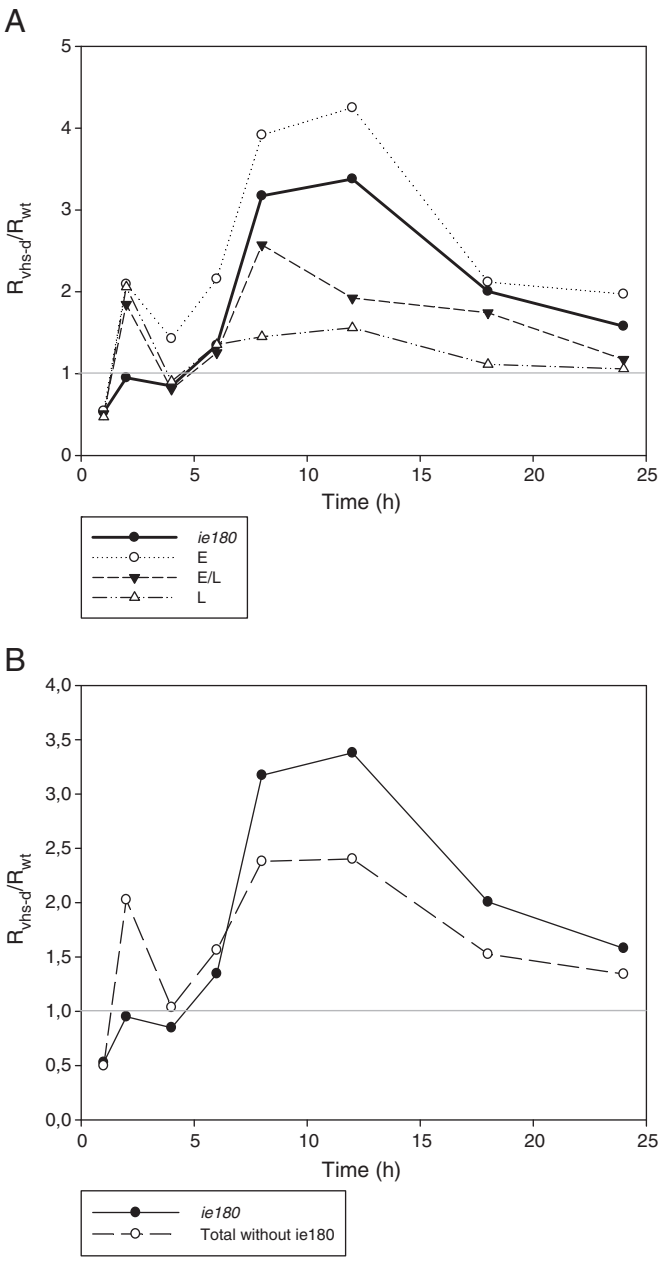

Fig. 6. Plot of the ratios of the levels of *ie180* transcript and the average the E, E/L and L transcripts ($\overline{R_r} = \overline{R}_{\Delta vhs} / \overline{R}_{wt}$). (A) This plot shows a high correlation in the changing of transcription kinetics in the *ie180* gene and in especially the E and E/L genes as a result of *vhs* mutation. (B) The change of the average kinetics of the total PRV genes is similar to that of *ie180* gene.

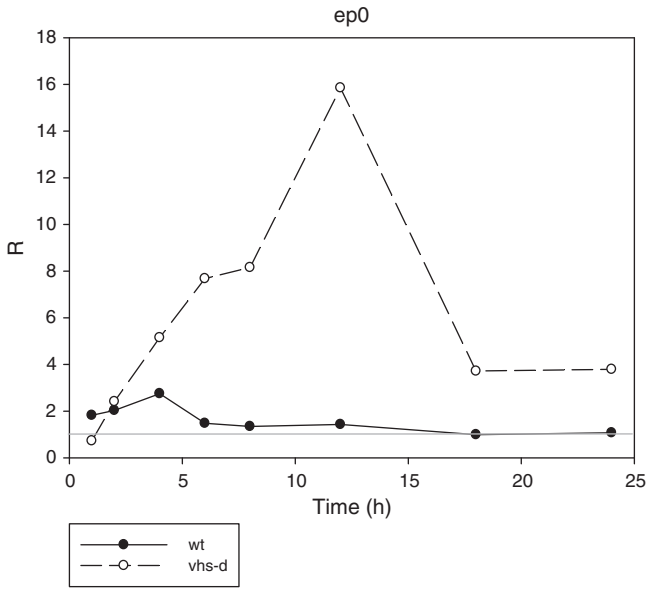

Fig. 7. Comparison of the expression kinetics of *ep0* transcripts in *vhsΔ* and *wt* viruses using the *R* values. It can be seen that the *vhs* gene-deletion has the greatest effect on the level of the *ep0* mRNAs. The difference is greatest at 12 hpi.

pRL494 vector. The unique *Nru*I site of this DNA segment was replaced by an *Eco*RI linker, which resulted in a frameshift mutation in the *ul41* gene (pUL41-RI). As a next step, a *lacZ* gene expression casette (containing the major IE1 promoter of HCMV, the lacZ gene and an SV40 polyA signal/termination sequences) bracketed by *Eco*RI linkers was inserted to the *Eco*RI site of pUL41-RI, generating pUL41-RI. This targeting plasmid was used for transfection along with purified viral DNA to porcine kidney-15 cells. Mutant viruses were selected on the basis of the blue plaque phenotype through multiple steps of plaque purification. Subsequently, the *lacZ* gene expression cassette was removed from the mutant virus by using a restriction cleavage transfection system, which was described earlier [26]. Isolation of the *lacZ*-containing viral DNA and its cleavage by *Eco*RI, was followed by ligation and cotransfection of the viral DNA. Mutant viruses lacking the *lacZ* gene cassette were isolated on the basis of the white plaque phenotype. The presence of mutation was verified by sequencing the PCR fragment containing the *Eco*RI fragment, which was amplified by using the DNA of the putative mutant virus. To confirm that solely the deleted gene was responsible for the altered phenotype of the virus, the mutant virus was rescued and analyzed for growth properties. The *vhs*-negative virus was tested for its spreading properties in a mouse model. The data show that the mutant virus exhibits a reduced speed of spreading in the mouse brain [10].

4.3. Infection conditions

The virus stock used for the experiments was prepared by infecting PK-15 cells with 10 plaque forming units (pfu)/cell, followed by incubation of the cells until a complete cytopathic effect was observed. To assess the effect of the *ul41* gene mutation on the PRV transcriptome, rapidly-growing semi-confluent PK-15 cells were infected with a high multiplicity of infection [MOI; 10 plaque forming units (pfu)/cell], using *wt* PRV or *vhs*-deleted virus, and incubated for 1 h, followed by removal of the virus suspension and washing of the cells with phosphate-buffered saline (PBS). Subsequently, new culture medium was added to the cells, which were further cultivated for 0, 1, 2, 4, 6, 8, 12, 18 or 24 h.

4.4. Isolation of total RNA

PK-15 cells (5×10^6 cells per flask) were washed in PBS and harvested for RNA purification. For quantitative real-time RT-PCR, total RNA was isolated from the cells with the NucleoSpin RNA II Kit (Macherey-Nagel GmbH and Co. KG) as recommended by the manufacturer. Briefly, cells were collected by low-speed centrifugation and lysed in a buffer containing the chaotropic salts, which inactivates RNases and allows nucleic acids to bind to silica membranes. Samples were treated with RNase-free rDNase solution (included in the Kit) to remove potential genomic DNA contamination. Subsequently, possible residual DNA contamination was removed by using Turbo DNase (Ambion Inc.). As a final step, RNA samples were eluted in RNase-Free Water (supplied with the Kit) in a total volume of 60 μl. RNA concentrations were measured in triplicate

spectrophotometrically, via the absorbance at 260 nm, in a BioPhotometer Plus (Eppendorf). The RNA solution was stored at −80 °C until use.

4.5. Reverse transcription

Isolated RNAs were reverse transcribed with gene-specific primers, using SuperScript III (Invitrogen) reverse transcriptase. Details of the reverse transcription (RT) reactions and the primer list were described previously [25]. Briefly, RT mixtures, containing total RNA, primer, SuperScript III enzyme, buffer, dNTP mix and RNasin RNase inhibitor (Promega), were incubated at 55 °C for 1 h. The first-strand cDNA amplifications were stopped by raising the temperature to 70 °C for 15 min. cDNAs were diluted 10-fold with Nuclease-Free Water (Promega Corp.) and stored at −80 °C until use.

4.6. Quantitative real-time PCR

SYBR Green-based (Absolute QPCR SYBR Green Mix, Thermo Scientific) quantitative real-time PCRs were carried out on the first-strand cDNAs in a real-time PCR cycler (Rotor-Gene 6000, Corbett Life Sciences), as published in our previous articles [25,27]. The accuracy of sampling and real-time RT-PCR analysis were ensured by using loading controls, as well as no-RT, no-primer, no-template controls (Supplemental Figure S3). Purified viral DNA were also used as a control to verify the specificity of the primers (Supplemental Figure S3).

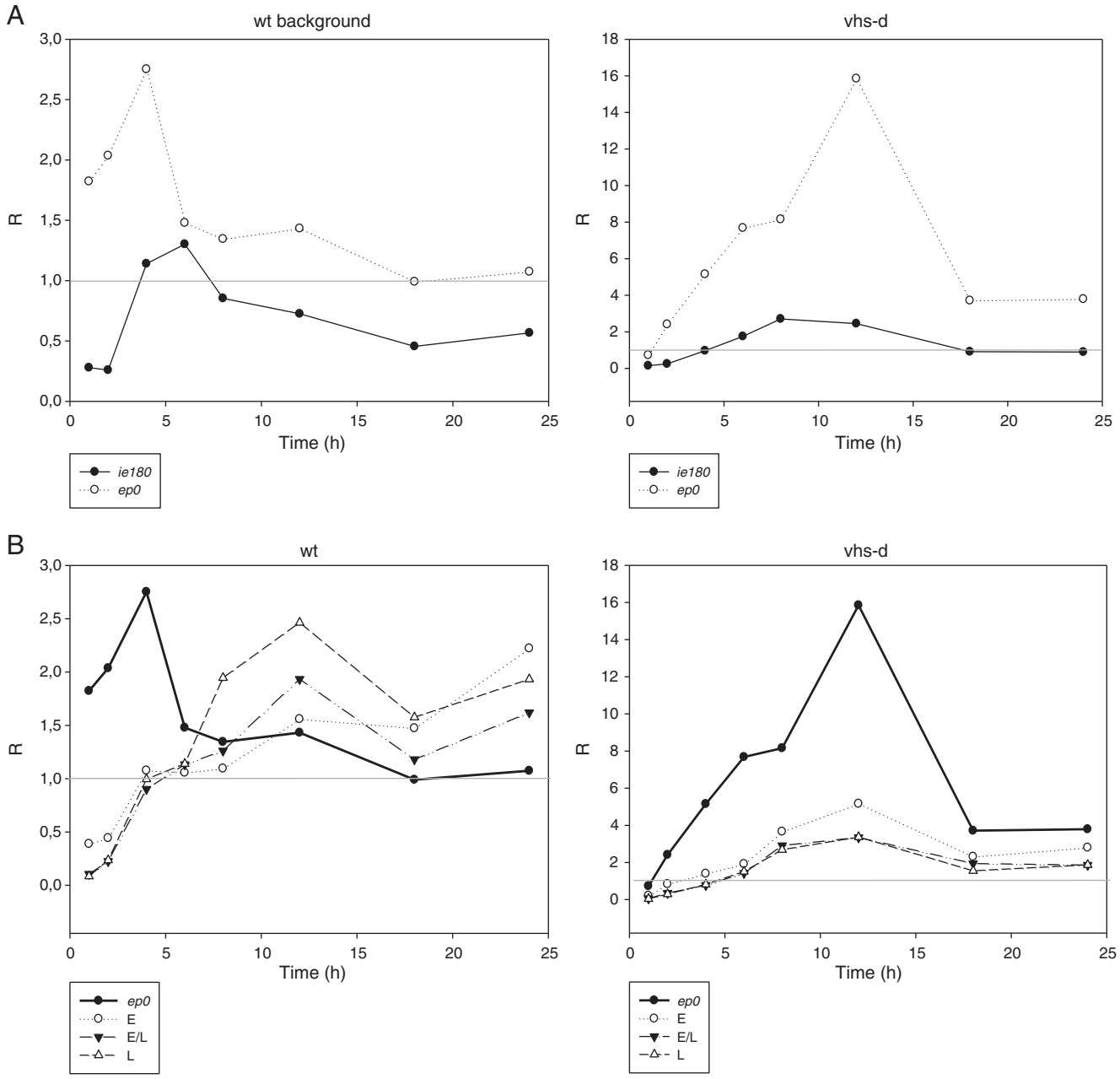

Fig. 8. Comparison of the expression kinetics of *ep0* gene with other PRV genes. (A) This diagram shows that the expression kinetics of the *ep0* and the *ie180* genes are uncorrelated in the *wt* background, whereas they are highly correlated in the *vhs*Δ virus. (B) The *ep0* gene expression is also highly correlated with those of other viral genes, independently of the kinetic classes to which the genes belong in the *vhs*Δ virus, which is not the case for the wt virus; (C) and the same is true for correlation of *ep0* gene with the average of total PRV genes.

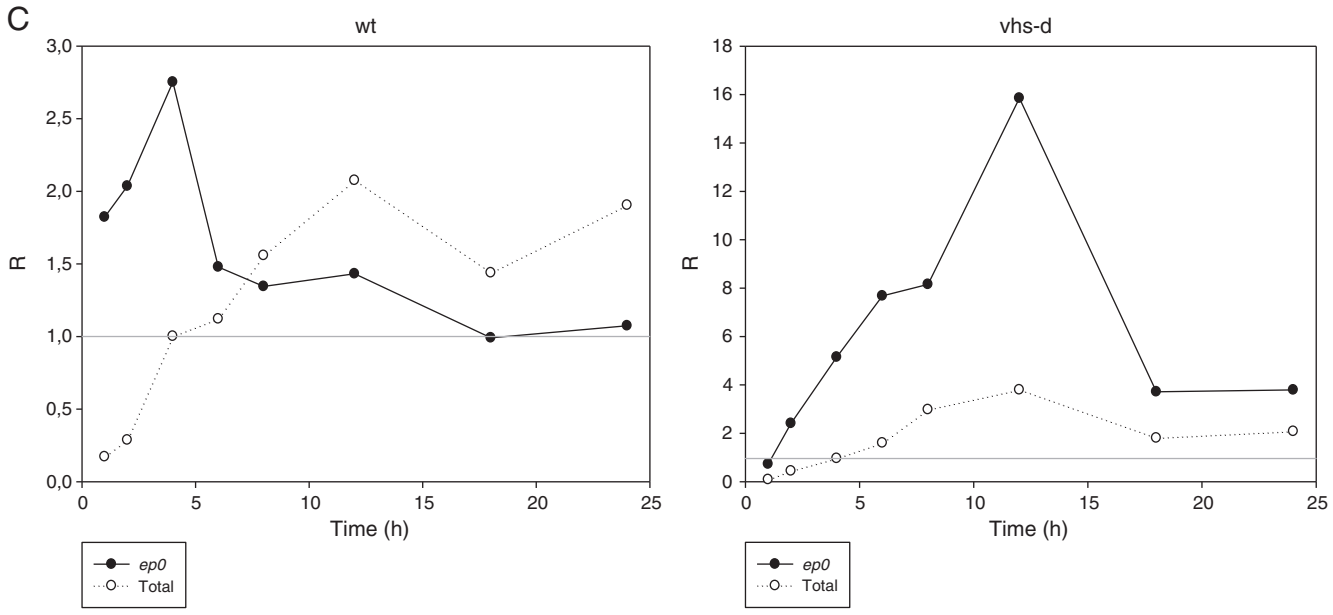

Fig. 8 (*continued*).

4.7. Data analysis and presentation

The relative expression ratios (R) were calculated via the following formula: $R = \frac{(E_{\text{sample 6h}})^{Ct_{\text{sample 6h}}}}{(E_{\text{sample}})^{Ct_{\text{sample}}}} : \frac{(E_{\text{ref 6h}})^{Ct_{\text{ref 6h}}}}{(E_{\text{ref}})^{Ct_{\text{ref}}}}$. The cDNAs were normalized to 28S cDNAs [25] using the Comparative Quantitation module of the Rotor-Gene 6000 software (Version 1.7.28, Corbett Research), which automatically calculates the efficiency of PCR reactions sample-by-sample, and automatically sets the thresholds. Furthermore, the average 6-h E^{Ct} values of the "samples" and the "references" were used as controls, as in our earlier publications [25,27]. The effect of the VHS protein on the gene expression was calculated by using the R_r values, the ratio between the R values of the *vhs*-knockout and the *wt* PRVs ($R_r = R_{vhs\Delta}/R_{wt}$), where $R_{\Delta vhs}$ and R_{wt} are the R values of a particular gene at a given time point in the *vhs*-knockout and *wt* 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 *wt* virus. Data were arranged on the basis of the kinetic classes of the genes (E, E/L and L) (Fig. 2) and were plotted graphically (Fig. 3). PRV genes were clustered in kinetic groups (IE, E, E/L and L) as in our earlier publications [25,27]. The net increase in the mRNA level (R_Δ) was calculated as shown earlier [25,27]. 28S RNA gene was used as a reference gene since ribosomal RNAs are not substrates of the VHS ribonuclease [17]. All data were analyzed by using the average and the standard deviance functions in Microsoft Excel. Moreover, we calculated the average R_r for the E, E/L and L genes for each time point: $\overline{R}_r = \overline{R}_{vhs\Delta} / \overline{R}_{wt}$ (Table 1). Pearson's correlation coefficient was calculated for the analysis of the gene expression kinetics, using the following formula [28]:

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

The Pearson correlation is a number between −1 and +1 that measures the linear relationship between two variables (labeled here as X and Y, which are the R values of two different genes or the average R values of genes that belong in the same kinetic class in the same time interval. $\overline{X}$ and $\overline{Y}$ are the average values, n is the sample number, and S_X and S_Y are the standard errors of the mean values for X and Y, respectively. A positive value for the correlation implies a positive association, while a negative value implies an inverse association.

Supplementary materials related to this article can be found online at doi:10.1016/j.ygeno.2011.03.010.

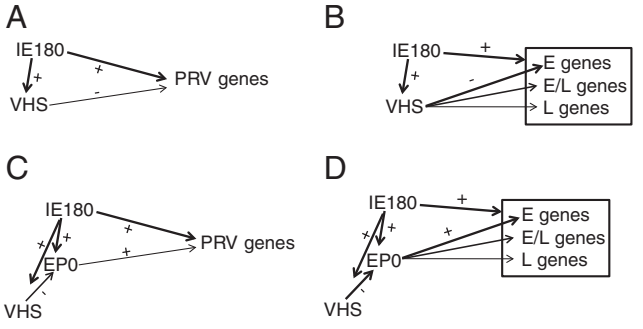

Fig. 9. A hypothesis as to how VHS proteins may affect the overall PRV gene expression. The IE180 transactivator controls the PRV gene expression in a stimulating manner. (A) Our data show that, except at 1 hpi, the VHS molecules exert a negative effect on the PRV mRNAs, and (B) especially those of the early gene transcripts. (C) We hypothesize that VHS may exert its major effects on PRV gene expression through regulation of the *ep0* transcripts. (D) According to this scenario, the EP0 transactivator is the major factor that selectively facilitates E gene expression in the early stage of viral infection.

Acknowledgments

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