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BRIEF COMMUNICATION

Synthesis and decay of varicella zoster virus transcripts

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Abstract Varicella zoster virus (VZV) is highly cell-associated. At least 68 VZV open reading frames (ORFs) are transcribed in varying amounts that increase as infection progresses. Using reverse transcriptase PCR, quantification of total and newly synthesized mRNA showed that ongoing VZV DNA replication is required for continued accumulation of VZV ORF 63, 9, and 40 transcripts. Analysis of stability of 4-thiouridine-labeled transcripts of nine VZV ORFs revealed a similar half-life for all VZV ORFs tested. Thus, difference in mRNA synthesis, and not mRNA decay, is the major factor contributing to the difference in the relative abundance of VZV transcripts in infected cells.

Keywords Varicella zoster virus · 4-Thiouridine · RNA synthesis · RNA decay · Newly synthesized RNA · Phosphonoacetic acid

Introduction

Varicella zoster virus (VZV) is a highly cell-associated neurotropic alpha-herpesvirus characterized in tissue culture by a particle/virion ratio approaching 40,000:1 (Carpenter et al. 2009). While cell-free VZV can be prepared by mechanical disruption of cells and low-speed centrifugation, virus yields seldom exceed one infectious VZV particle per 500 infected cells (Cole and Grose 2003). The low yield of cell-free virus has significantly hindered molecular studies of the virus. For example, while at least 68 unique VZV genes are expressed during productive VZV infection in tissue culture (Cohrs et al. 2003; Kennedy et al. 2005; Nagel et al. 2009), little is known about the kinetics of VZV gene transcription. Recently, we reported a technique to metabolically label newly synthesized RNA with 4-thiouridine (4sU) to facilitate its extraction from total RNA (Dölken et al. 2008). This technique exploits the cells' ability to incorporate 4sU into newly synthesized RNA during transcription. The newly synthesized RNA molecules are distinguished from unlabeled RNA synthesized before or after 4sU labeling by the presence of thiol moieties, which facilitate subsequent separation of labeled and unlabeled RNA using affinity chromatography. Metabolic labeling of newly synthesized RNA with 4sU has been tested extensively to exclude the possibility of any significant effect of 4sU on cell gene transcription (Kenzelmann et al. 2007, Dölken et al. 2008). Herein, we applied 4sU labeling of RNA synthesized in VZV-infected melanoma (MeWo) cells to determine the abundance of newly synthesized viral transcripts during VZV infection, to show the effect of viral DNA replication on VZV mRNA synthesis, and to calculate VZV mRNA decay rates. An understanding of the kinetics of VZV gene transcription will aid in the analysis of productive virus growth and factors involved in the establishment of latency.

Results

To validate the 4sU-labeling procedure in the context of VZV infection, MeWo cell cultures were infected with

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VZV by co-cultivation. At 48 h post-infection (hpi), samples were labeled with 500 μM 4sU for 1 h or left untreated, followed by immediate RNA extraction. Extracted RNA was biotinylated and separated on streptavidin affinity column. RNA samples before (input) and after (output) affinity separation were resolved on non-denaturing agarose gels (Fig. 1). The input RNA samples isolated from both 4sU-treated and untreated cells showed a similar pattern of RNA with discrete 18S and 28S rRNA bands, indicating that the quality of input RNA was not affected by 4sU treatment. The output RNA sample isolated from 4sU-treated cells showed a heterogeneous complex of high-molecular-weight RNA with no typical rRNA bands, and the output RNA sample isolated from untreated cells did not contain RNA. Thus, newly synthesized RNA in VZV-infected MeWo cells was successfully separated from preexisting, unlabeled RNA.

While no RNA was detected by agarose gel electrophoresis in the output RNA sample from untreated cells, the possibility remained that small amount of unlabeled RNA still was present. To address this possibility, multiple MeWo cell cultures (n=8) were infected with VZV, and total RNA was extracted 24–72 hpi without 4sU labeling. Following biotinylation and affinity separation, input and output RNA samples were reverse-transcribed and analyzed by quantitative real-time PCR (qPCR) targeting VZV ORFs 9, 40, 63, and cell GAPdH sequences. The choice of VZV targets ensured representation of ORFs belonging (by homology to HSV-1 genes) to the immediate-early (ORF63), early (ORF9), and late (ORF40) kinetic classes. The cycle threshold (C_T) values determined by qPCR and normalized to the same amount of input RNA were used to calculate the difference in C_T values (ΔC_T) between corresponding input and output RNA samples. The average ΔC_T±95% confidence interval was 16.78±1.57 (ORF9), 13.94±0.83 (ORF40), 13.32±1.23 (ORF63), and 15.04±1.02 (GAPdH). Since the C_T values were normalized to the amount of RNA within each assay, the fold difference for each transcript in the input RNA and output RNA samples were calculated (Livak and Schmittgen 2001) and expressed as the copy number of newly synthesized transcripts per 10^6 transcripts in the initial input RNA. Using these calculations, we found that the amount of nonspecific background was negligible: 9 copies of VZV ORF9, 64 copies of VZV ORF40, 98 copies of VZV ORF63, and 30 copies of cellular GAPdH RNA molecules per 10^6 molecules of input transcripts.

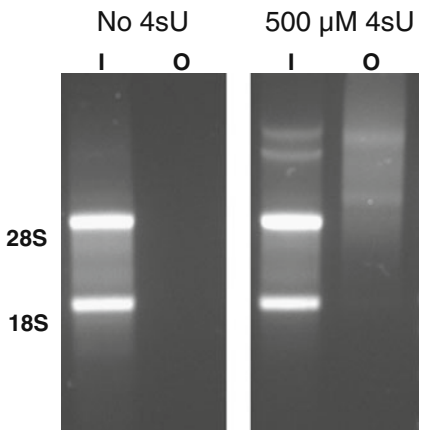


Fig. 1 Size profile of input and output RNA isolated from 4sU-labeled and unlabeled VZV-infected MeWo cells. Input (*I*) and output (*O*) RNA samples extracted from cells treated for 1 h in either the medium without or with 500 μM 4sU was resolved on 1% agarose non-denaturing gels. The 28S and 18S rRNA bands are labeled

The relationship between VZV gene transcription and virus DNA replication was investigated. Five groups of MeWo cell cultures (three biological replicates per group) were infected with VZV. The first group was labeled with 4sU from 23 to 24 hpi followed by total RNA extraction. At 24 hpi, the culture medium in the remaining four groups was removed and replaced with fresh medium with or without 50 μg/ml phosphonoacetic acid (PAA). PAA, an inhibitor of herpesvirus DNA replication, was used at the minimal concentration required to prevent plaque formation in VZV-infected cells (May et al. 1977). PCR analysis of DNA extracted from VZV-infected MeWo cells that had been treated with 50 μg/ml PAA revealed complete inhibition of virus DNA synthesis (Fig. 2). The cultures were then incubated for 2, 5, 23, or 47 h, labeled with 4sU

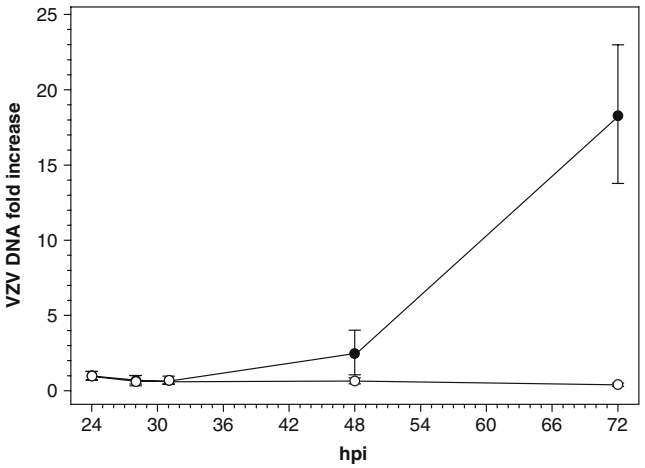


Fig. 2 Inhibition of VZV DNA synthesis by PAA. VZV-infected MeWo cells were treated with 50 μg/ml PAA (*open circles*) beginning at 24 hpi or untreated (*filled circles*). At times indicated, DNA was extracted and 100 ng DNA was analyzed by qPCR with VZV- or GAPdH-specific primers/probes. The increase in VZV DNA copies relative to the start of PAA treatment was normalized to cell DNA (GAPdH). In samples not treated with PAA, the relative abundance of VZV DNA copies increased as the infection progressed. However, in PAA-treated, VZV-infected MeWo cells, virus replication was completely inhibited. Data represent the means and 95% confidence intervals of three biological replicates

for 1 h, and total RNA was extracted at 27, 30, 48, or 72 hpi. The final time point was selected based on the observation that the virus-induced cytopathic effect was maximal at 72 hpi, after which time the cells begin to deteriorate and detach. Trizol-extracted DNase-treated RNA was biotinylated, affinity-purified, and the resulting input and output RNA samples reverse-transcribed and analyzed by qPCR for VZV ORFs 9, 40, 63, and cell GAPdH sequences.

The abundance of VZV ORF 9, 40, and 63 transcripts, when normalized to equal amounts of cellular GAPdH transcripts, increased in VZV-infected MeWo cells after infection (Fig. 3a, c, e). At each time point, the number of preexisting (filled circles) and newly synthesized (open circles) transcripts increased at a similar rate. Numerical analysis revealed that at 24 hpi, 1 μg of RNA contained $6.37\pm1.96\times10^7$ total copies of VZV ORF9, $4.19\pm2.75\times10^6$ total copies of VZV ORF40, and $2.58\pm0.67\times10^7$ total copies of VZV ORF63 transcripts, of which $4.82\pm0.62\times10^5$ copies of ORF 9, $1.83\pm0.18\times10^4$ copies of ORF40, and $2.34\pm0.62\times10^5$ copies of ORF63 were synthesized in the 1 h between 23 and 24 hpi. Therefore, the proportion of VZV transcripts synthesized between 23 and 24 hpi to total transcripts was 0.76±0.25% for ORF9, 0.44±0.29% for ORF40, and 0.91±0.34% of ORF63. By 72 hpi, the abundance of transcripts corresponding to VZV ORFs 9, 40, and 63 increased by 8.03±3.12-, 19.81±7.38-, and 11.24±4.20-fold, respectively. At this time, the proportion of newly synthesized VZV transcripts to total was 0.33±0.16%, 0.36±0.20%, and 0.82±0.30% for VZV ORFs 9, 40, and 63, respectively.

When VZV DNA replication was inhibited by PAA, a continuous increase in the abundance of VZV transcripts was not seen (Fig. 3b, d, f). In fact, the abundance of newly synthesized transcripts in PAA-treated cells decreased from 48 to 72 hpi. The decrease in the abundance of newly synthesized transcripts per 1 μg of input RNA was $4.25\pm2.46\times10^5$ (ORF9), $2.17\pm0.48\times10^4$ (ORF40), and $2.29\pm0.36\times10^5$ (ORF63).

Consistent with previous reports, the relative abundance of ORF9 transcripts was the highest and ORF40 was the lowest among the three VZV transcripts examined at each time point (Cohrs et al. 2003). On average, the ratio of ORF9 to ORF63 transcripts was 2.15±0.18, and the ratio of ORF63 to ORF40 was 8.86±0.91.

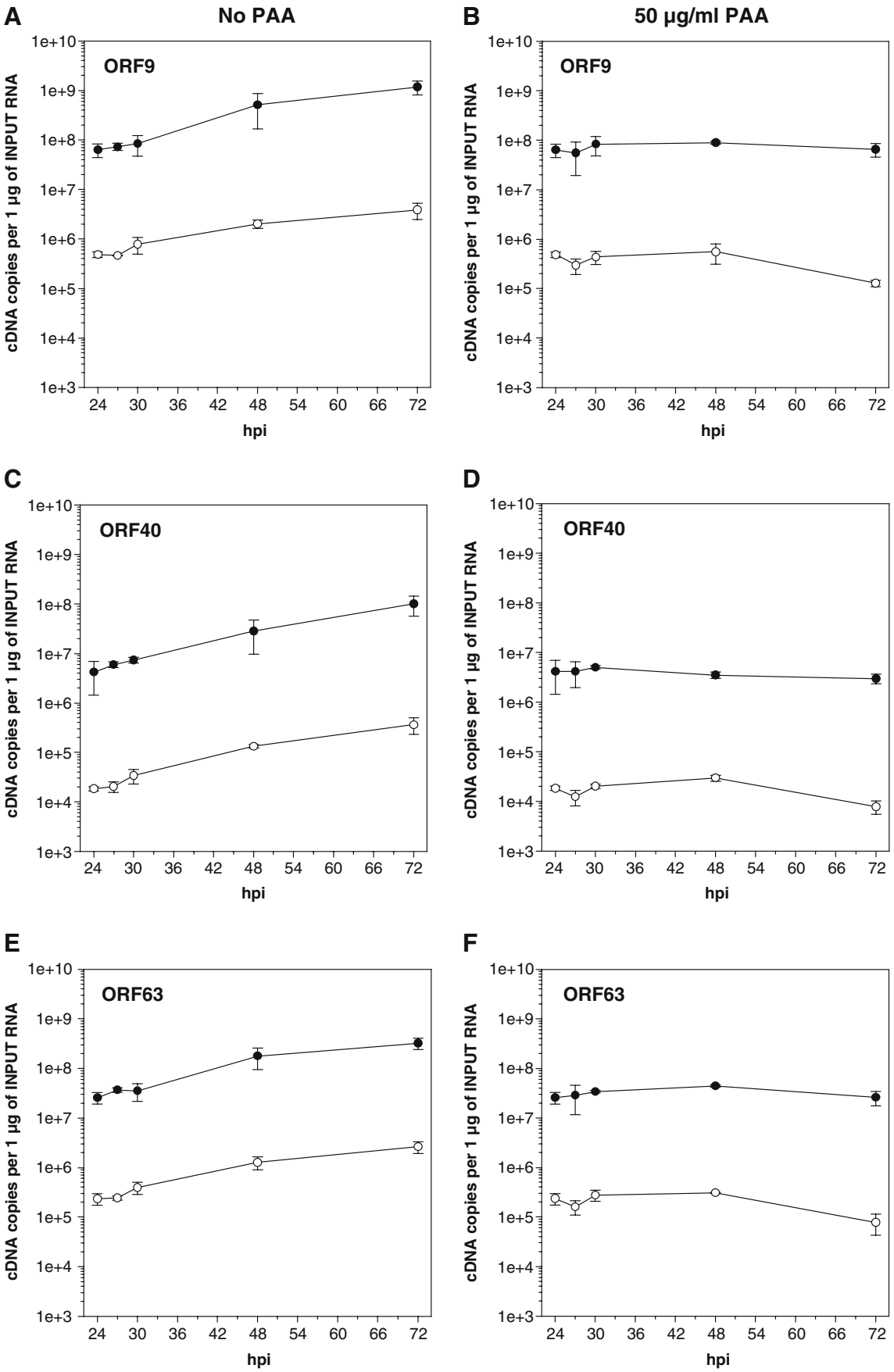
In addition to studying the kinetics of VZV RNA synthesis, 4sU labeling provides a simple, non-radioactive PCR-compatible method to analyze virus transcript stability. VZV-infected MeWo cell cultures were incubated for 47 h, and RNA was labeled with 4sU between 47 and 48 hpi. After the 1-h 4sU-labeling period, the cultures were incubated in Dulbecco's minimal essential medium (DMEM) without 4sU, and RNA was extracted at 48, 54, 60, and 72 hpi and affinity-purified. C_T values were determined by RT-qPCR for immediate-early (ORFs 61, 62, 63), early (ORFs 9, 29, 31, 66), and late (ORFs 21, 40) VZV transcripts, and the results were normalized to the average C_T value for GAPdH transcripts at each time point. Analysis of the resultant C_T values provides a sensitive measure of the decay rate for each virus transcript. Figure 4a shows the relationship between the amounts of 4sU-labeled RNA present as a function of hours after pulse labeling and indicates that each VZV transcript decayed at the same rate. The half-lives of VZV ORFs 9, 21, 29, 31, 40, 61, 62, 63, and 66 transcripts ranged from 2.91±0.36 to 3.69±0.48 h and were not significantly different (overlapping 95% CI; Fig. 4b). The corresponding dissociation constants ranged from 0.188±0.025 to 0.238±0.029 h^{-1} and were also not significantly different.

Discussion

In summary, we demonstrated the utility of metabolic labeling of newly synthesized RNA with 4sU to study both the accumulation and degradation of VZV transcripts. While a number of techniques to achieve these goals exist (reviewed in Ross 1995), the present method is compatible with quantitative PCR analysis because newly synthesized transcripts are physically separated from preexisting transcripts. We observed an increase in the abundance of VZV ORF 9, 40, and 63 transcripts in virus-infected MeWo cells between 24 and 72 hpi, which is in agreement with macroarray data showing a uniform and continuous increase in transcript abundance for 68 unique VZV ORFs within the same time frame (Cohrs et al. 2003). In addition, this uniform and continuous increase in VZV immediate-early, early, and late gene transcripts was extended to newly synthesized transcripts, indicating that when virus is propagated by co-cultivation, the infection is non-synchronous. Taken together, these observations demonstrate that the synthesis of VZV transcripts continues in cell cultures for at least 3 days post-infection.

Treating VZV-infected cells with PAA prevented any increase in the synthesis of VZV DNA or viral transcripts, suggesting that newly synthesized viral DNA serves as a template for transcription. PAA inhibition of VZV DNA synthesis not only prevented further accumulation of ORF 9, 40, and 63 transcripts but also resulted in a decreased synthesis of these transcripts at late times post-infection. The most likely explanation for the decrease in newly synthesized virus transcripts is VZV DNA encapsidation, which makes the virus DNA templates inaccessible for transcription.

We also measured the decay of VZV transcripts, which, to our knowledge, is the first report on VZV transcript stability. For all VZV ORFs studied, the half-lives and associated dissociation constants were remarkably similar,


A
No PAA
ORF9
B
50 µg/ml PAA
ORF9
C
ORF40
D
ORF40
E
ORF63
F
ORF63
cDNA copies per 1 µg of INPUT RNA
1e+10
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
24 30 36 42 48 54 60 66 72
hpi

◀ **Fig. 3** Effect of VZV DNA synthesis on the relative abundance of VZV ORFs 9, 40, and 63 transcripts in VZV-infected MeWo cells. Total RNA was extracted at 24, 27, 30, 48, or 72 hpi from VZV-infected MeWo cells grown in a medium lacking PAA (**a**, **c**, **e**) or containing 50 μg/ml PAA (**b**, **d**, **e**). After biotinylation and affinity purification, input RNA (*filled circles*) and output RNA (*open circles*) RNA samples were analyzed by qPCR targeting VZV ORFs 9 (**a**, **b**), 40 (**c**, **d**), and 63 (**e**, **f**). Data represent the means and 95% confidence intervals of three biological replicates

suggesting that a difference in mRNA synthesis, rather than in mRNA degradation, is the major factor in determining VZV transcript abundance in infected cells. In addition, we observed no correlation between the stability of VZV transcripts and the kinetic class of its cognate gene. For example, transcripts classified as immediate-early (VZV ORFs 61, 62, 63), early (VZV ORFs 9, 29, 31, 66), and late (VZV ORF 21, 40) based on their homology to HSV-1 or on the data reported elsewhere (Cohen 2010) were all equally stable. The observation that the VZV transcript stability does not depend on the kinetic class is in agreement with Oroskar and Read (1989) who determined that the half-lives for HSV-1 immediate-early (ICP0, ICP4, ICP27), early (UL23, UL30, ICP8), and late (ICP5, UL22, UL27, UL44) transcripts ranged from 1 to 2.5 h and were not significantly different.

Materials and methods

Cells and virus

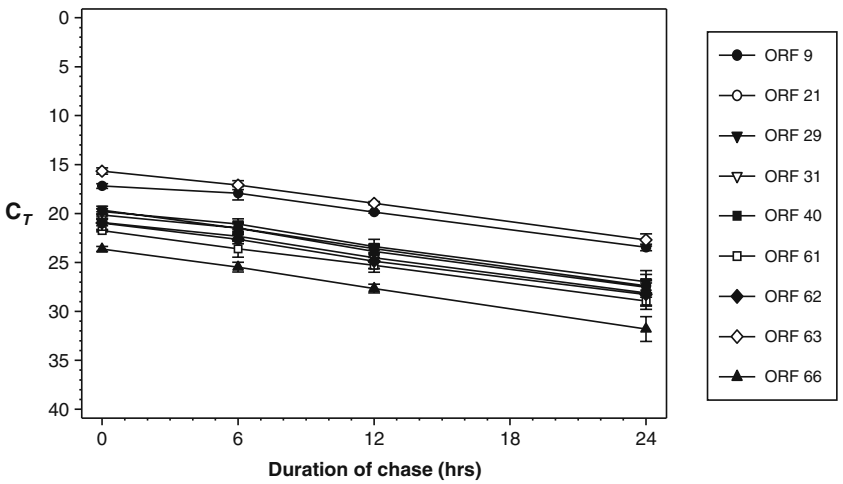
MeWo cell cultures were maintained in DMEM (Sigma, St Louis, MO) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. VZV strain 961 used in this study was derived from parental Oka strain and maintained as a BACmid (Tischer et al. 2007). The cells were infected by co-cultivation of uninfected and VZV-infected cells at an infected/uninfected cell ratio of 1:10. Cell-associated VZV was selected as a source of infection because the viral titers typically achieved for cell-free VZV were insufficient for a consistent detection of newly synthesized viral transcripts.

Metabolic labeling of newly synthesized RNA

The procedure for 4sU labeling, extraction, biotinylation, and affinity purification of newly synthesized RNA (Dölken et al. 2008) was followed with minor modifications. Briefly, the cells were labeled by incubating for 1 h in the medium containing 500 μM 4sU (Sigma). Total RNA was extracted in 5 ml TRI reagent (Ambion, Austin, TX), isopropanol-

Fig. 4 Decay of VZV transcripts in VZV-infected MeWo cells. At 47 hpi, VZV-infected MeWo cells were pulse labeled 4sU for 1 h. At the indicated times post-labeling, 4sU-labeled RNA was extracted and analyzed by RT-PCR. Cycle threshold (C_T) values were determined for VZV ORF 9, 21, 29, 31, 40, 61, 62, 63, and 66 transcripts and were plotted as a function of time post-4sU labeling (**a**). Half-lives and corresponding dissociation constants were calculated for each VZV transcript analyzed (**b**). Data represent the means and 95% confidence intervals of three independent experiments

A



B

Transcript	mRNA half-life (hr)		mRNA decay constant (hr^{-1})	
ORF9	3.69	0.48	0.188	0.025
ORF21	3.26	0.44	0.213	0.029
ORF29	3.26	0.48	0.213	0.032
ORF31	3.27	0.56	0.212	0.037
ORF40	3.02	0.34	0.229	0.026
ORF61	3.35	0.40	0.207	0.025
ORF62	3.25	0.57	0.213	0.038
ORF63	3.37	0.31	0.206	0.019
ORF66	2.91	0.36	0.238	0.029

precipitated, dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.4), and treated with DNAse as described (Cohrs et al. 1994). RNA was recovered after DNAse treatment by affinity chromatography (RNeasy RNA Mini-kit, Qiagen, Valencia, CA). DNase-treated RNA was biotinylated in reactions consisting of 100 μg/ml RNA and 200 μg/ml *N*-(6-(biotinamido)hexyl)-3′-(2′-pyridyldithio)-propionamide (biotin-HPDP, Thermo Scientific, Rockford, IL) in TE buffer. Samples were rotated at 24 rpm for 2 h at room temperature, followed by two extractions with chloroform/isoamyl alcohol (24:1, *v*/*v*). RNA was precipitated in 0.2 M NaCl and 50% isopropyl alcohol, collected by centrifugation (4°C, 20 min, 12,000×*g*), and dissolved in TE buffer (INPUT RNA). Streptavidin-coated paramagnetic beads (100 μl; Miltenyi Biotech) were added to input RNA (40–80 μg) in 100 μl TE. Samples were rotated (24 rpm, 15 min, room temperature) and applied to magnetic columns (Miltenyi) equilibrated with 1 ml wash buffer (1 M NaCl, 0.1% Tween-20 in TE). The columns were washed three times with wash buffer at 65°C and three times with wash buffer at room temperature. RNA was eluted in two 100-μl aliquots of freshly prepared 100 mM DTT in lysis buffer (RLT, Qiagen). Importantly, the two DTT elutions were separated by 3-min intervals. RNA was diluted with 500 μl 100% ethanol, extracted by affinity chromatography (RNeasy MinElute, Qiagen), washed once with RPE buffer, then with 80% ethanol, and eluted in 30 μl RNase-free water (output RNA).

Reverse transcription qPCR

The input and output RNA samples were reverse-transcribed in 20-μl reactions as described (Cohrs and Gilden 2007). Briefly, 10–50 ng RNA was incubated with 2.5 μM dN(dC/dA/dG)dT_{18} primer at 65°C for 10 min and 4°C for 10 min, after which the RNA was incubated in a final concentration of 1 mM each dNTP, 20 U RNase inhibitor, and 10 U

Table 1 qPCR primers and probes

Name	Sequence (5′–3′)	5′ location[a]
ORF 9—forward	GGGAGCAGGCGCAATTG	11443
ORF 9—probe	CAATTGCCAGCGGGAGACC	11454
ORF 9—reverse	TTTGGTGCAGTGCTGAAGGA	11496
ORF21—forward	TGTTGGCATTGCCGTTGA	32816
ORF21—probe	CTGCTTCCCCAGCACGTCCGTC	32835
ORF21—reverse	ATAGAAGGACGGTCAGGAACCA	32881
ORF29—forward	GGCGGAACTTTCGTAACCAA	52952
ORF29—probe	TCCAACCTGTTTTGCGGCGGC	52973
ORF29—reverse	CCCCATTAAACAGGTCAACAAAA	53017
ORF31—forward	CACAAAAACACCCGACTCGAA	58263
ORF31—probe	TACCAGATCCCGACGAAGCGTGC	58285
ORF31—reverse	GATGAGGTGGTTGTTATTGTTCTATTG	58350
ORF40—forward	ACTTGGTAACCGCCCTTGTG	72516
ORF40—probe	ATGGGAAAGGCCGTCCGCGG	72536
ORF40—reverse	CGGGCTACATCATCCATTCC	72573
ORF61—forward	TCCGACGACGATGGTTTTTC	104242
ORF61—probe	CTGAGCCGTCATTTGAAG	104223
ORF61—reverse	CCGGTAGGATGTCGATGGAA	104185
ORF62—forward	CCTTGGAAACCACATGATCGT	106985
ORF62—probe	TGCAACCCGGGCGTCCG	107007
ORF62—reverse	AGCAGAAGCCTCCTCGACAA	107063
ORF63—forward	GCTTACGCGCTACTTTAATGGAA	110939
ORF63—probe	TGTCCCATCGACCCCCTCGG	110969
ORF63—reverse	GCCTCAATGAACCCGTCTTC	111005
ORF66—forward	CCACGTTACCGAACAGATTTATACTG	113520
ORF66—probe	TGGACATATGGAGTGCCGGGATTGTA	113550
ORF66—reverse	CTAGCTGCAAAGCGCAACCTCCCC	113602
GAPdH—forward	CACATGGCCTCCAAGGAGTAA	1048
GAPdH—probe	CTGGACCACCAGCCCCAGCAAG	1074
GAPdH—reverse	TGAGGGTCTCTCTCTTCCTCTTGT	1122

[a] VZV sequences located on GenBank ID: X04370; GAPdH sequences located on GenBank ID: BC013310.

reverse transcriptase (Roche, Indianapolis, IN) in 1× reaction buffer (50 mM Tris–HCl, 30 mM KCl, 8 mM $MgCl_2$, pH 8.5) at 50°C for 60 min, 85°C for 5 min, and 4°C for 10 min. The resulting cDNA was analyzed by qPCR as described (Cohrs and Gilden 2007). The primers and probes used for qPCR are listed in Table 1. The cycle threshold (C_T) values were obtained and used to calculate the absolute and relative abundance of transcripts. The absolute abundance of VZV DNA and cDNA was determined by comparing their C_T values with those obtained using serial $\log_{10}$ dilutions of BACmid DNA containing the entire VZV genome which was included in duplicates in every PCR plate. A BACmid-derived positive control containing10^6 copies per reaction of full-length VZV genome was included when the standards were not used. A no-template control was also included with all runs. All samples were analyzed in triplicate.

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Conflict of interest The authors declare that they have no conflict of interest.

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