Mapping the Temporal Transcriptomic Signature of a Viral Pathogen through CAGE and Nanopore sequencing

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**Abstract**

**Introduction**

Equid alphaherpesvirus 1 (EHV-1), a veterinary pathogen belonging to the Varicellovirus genus, is responsible for significant economic losses in the global equine sector. This research involved timescale gene expression profiling and transcriptional reannotation of this herpesvirus.

**Methods**

We employed cap analysis gene expression sequencing (CAGE-Seq) on Illumina platform to determine the transcript start sites alongside long-read direct cDNA sequencing (dcDNA-Seq) on Oxford Nanopore Technology platform to detect full-length viral transcripts. Samples were collected at nine distinct stages of the viral lifecycle, with triplicates taken at each stage. We also applied protein synthesis inhibition to determine the immediate-early gene expression of the virus. Earlier data on native RNA sequencing was also utilized to validate the results. The sequencing data were processed using the LoRTIA and NAGATA software tools.

**Results**

The time-course analysis of viral transcript expression using long-read dcDNA-Seq enabled the characterization of these transcripts based on their kinetic behavior throughout the replication cycle. Furthermore, the study involved a comprehensive reannotation of the EHV-1 transcriptome. CAGE-Seq helped identify the transcription start sites and promoter regions, while dcDNA-Seq provided a more accurate approach to capturing full-length transcripts and isoform diversity. Through an integrated approach, we identified and validated 170 novel putative transcripts, thereby refining the EHV-1 transcriptome annotation. These methods allowed for a more detailed and accurate mapping of the EHV-1 transcriptome, uncovering previously unknown transcripts and refining the existing annotations.

**Conclusions**

The shifting patterns in transcript isoforms and overlaps suggest a sophisticated regulatory network that enables EHV-1 to precisely modulate gene expression throughout its replication cycle. The presence of multiple isoforms per gene indicates that the virus can adapt to different stages of infection by producing a variety of transcripts. This likely enhances its genomic efficiency and allows it to respond more effectively to the host's environment.

**Introduction**

Equid alphaherpesvirus 1 (EHV-1), also referred to as *Varicellovirus equidalpha1* [1] commonly presents with symptoms such as upper respiratory tract disease, spontaneous abortion in pregnant mares, neonatal death, and life-threatening myeloencephalopathy [2]. EHV-1 contains an approximately 150 kilobase pair double-stranded DNA genome. This genome is organized into two segments designated as unique short (US) and unique long (UL) regions, both surrounded by inverted repeats (IRs) [3,4]. The complete viral genome contains 80 open reading frames (ORFs) [5] among which five genes (ORF1, 2, 67, 71, and 75) are absent in other alphaherpesviruses with annotated genomes [6]. Given that four ORFs are situated in the IR region, the EHV-1 genome comprises a total of 76 unique protein-coding genes. We note that the term 'ORF' is used to denote the entire genes in EHV-1, not just the protein-coding parts. Similar to other alphaherpesviruses, EHV-1 can either productively infect cells or enter a latent state in specific peripheral neurons [7].

EHV-1 genes fall into three categories: immediate-early (IE), early (E), and late (L). The regulation of these genes is governed by viral transcription factors, specifically ORF5, 12, 63, 64, and 65 [8,9]. Notably, EHV-1 has a sole IE gene [10], ORF64, which bears homology to *rs1* gene of herpes simplex virus type 1 (HSV-1) coding for the ICP4 protein. Most of the viral E genes encode enzymes required for DNA replication, whereas the L genes specify structural proteins found in the virion, such as capsid and spike proteins. Late genes are further subdivided into leaky late (L1) and true late (L2), depending on their reliance on DNA replication for their expression [10].

Long-read sequencing (LRS), developed by Pacific Biosciences (PacBio) through synthesis-based sequencing and by Oxford Nanopore Technologies (ONT) through nanopore sequencing, has become essential in modern transcriptome analysis. The long-read RNA sequencing (lrRNA-Seq) technique is particularly effective in identifying transcription start sites (TSSs), transcription end sites (TESs), splice sites, alternative splicing, embedded transcripts, multigenic RNA molecules, and transcriptional overlap [11]. While lrRNA-Seq platforms can deliver full-length cDNA or native RNA sequences, they come with reduced throughput and a higher incidence of sequencing errors compared to short-read sequencing (SRS) platforms [12–17]. In transcriptomics, inaccuracies in sequencing are not a significant concern when the genomic sequence of a specific organism is well-established. The lrRNA-Seq technology based on ONT is highly suitable for native RNA sequencing. It guarantees correct read orientation and is free from the artefacts generated by reverse transcription and PCR [11,18], and it also facilitates the detection of RNA modifications [19].

Transcriptomic studies in herpesviruses have applied both PacBio and ONT sequencing methods [15,17,20–26]. Temporal dynamics of herpesvirus gene expression has also been examined using SRS [27]. Our previous transcriptome profiling study, which employed a native RNA sequencing approach, identified the canonical EHV-1 transcripts, including mRNAs, non-coding RNAs (ncRNAs) and long multi-gene transcripts [28]. However, direct RNA sequencing (dRNA-Seq) has limitations in accurately identifying TSSs due to 5'-truncation caused by motor protein stalling during sequencing. To address this and refine the EHV-1 transcriptome annotation, we integrated cap analysis of gene expression sequencing (CAGE-Seq) with direct cDNA sequencing (dcDNA-Seq). CAGE-Seq provides high-resolution TSS mapping, while dcDNA-Seq captures full-length transcripts without the 5'-truncation issues of dRNA-Seq. This combined approach allowed us to validate transcripts previously identified but excluded due to strict filtering criteria and to discover additional transcript isoforms with greater accuracy. Using dcDNA-Seq, we sequenced 27 samples collected at nine time points spanning 1 to 48 hours post-infection, with three replicates taken at each time point. This extensive temporal sampling captured the full dynamics of viral gene expression across the complete infection cycle. By analyzing this time-resolved data, we clustered genes into *de novo* kinetic classes based on their expression dynamics. Additionally, reanalyzing the data with integrated CAGE-Seq refined existing annotations, validated previously excluded transcripts, and identified previously unrecognized transcript isoforms with greater accuracy.

## **Methods**

## **Cells and viruses**

In this study, we utilized the field isolate *equid alphaherpesvirus 1* strain MdBio (EHV-1-MdBio), which was originally isolated from the organs of an aborted colt fetus in the 1980s at Marócpuszta, Hungary, and has been previously described [28]. The virus was propagated in a confluent rabbit kidney (RK-13) epithelial cell line (ECACC: 00021715). Cells were cultivated in DMEM (Sigma), supplemented with 10% fetal calf serum and 80 μg of gentamycin per ml (Gibco) at 37 °C in the presence of 5% CO2. For the preparation of virus stock solution, cells were infected with 0.1 multiplicity of infection [MOI = plaque-forming units (pfu)/cell]. Viral infection was allowed to progress until complete cytopathic effect was observed. As a next step, three successive cycles of freezing and thawing of infected cells were carried out to release of viruses from the cells. For the sequencing experiments, RK-13 cells were infected with 4 MOI of EHV-1-MdBio in three technical replicates. Infected cells were incubated for 1 h at 4 °C, followed by removal of the virus suspension and washing the cells with phosphate-buffered saline. As a next step, new culture medium was added to the infected cells, which were incubated for 1, 2, 4, 6, 8, 12, 18, 24, or 48 h. After the incubation, the culture medium was removed, and the infected cells were frozen at −80 °C until further use.

**Cycloheximide treatment of cells**

RK-13 cells were grown in DMEM supplemented with 10% fetal bovine serum until they reached 60–70% confluency. The medium was then replaced with 5 mL of serum-free DMEM containing either 20 or 100 μg/mL cycloheximide (CHX). After a 1-hour incubation, this medium was substituted with 2 mL of a 10 MOI virus solution, also containing the same CHX concentrations, and the cells were incubated for either 6 or 8 hours. Post-incubation, the CHX-treated cells were washed once with PBS, scraped off the dish, and centrifuged at 2000 g for 2 minutes. Following the removal of the supernatant, the cell samples were immediately placed on dry ice for future analysis.

## **RNA extraction**

The RNA extraction was conducted using the NucleoSpin RNA kit from Macherey-Nagel. The process began by lysing the cells in a buffer containing chaotropic ions to deactivate RNases. This step facilitated the binding of DNA and RNA molecules to the silica membrane. To eliminate any residual genomic DNA (gDNA), all samples were treated with DNase I. The total RNA was then eluted in nuclease-free water. Further purification to remove any remaining gDNA was achieved using the TURBO DNA-free™ Kit from Invitrogen. The RNA concentration in the samples was determined using the Qubit 4.0 fluorometer and the Qubit Broad Range RNA Assay Kit, also from Invitrogen (**Supplementary Table S1**). Quality control was performed with the Agilent TapeStation 4150, and only samples with RIN scores equal to or greater than 9.2 were used for cDNA synthesis and subsequent experiments.

**Purification of polyadenylated RNA**

The poly(A)+ RNA fraction was extracted from the total RNA using the Oligotex mRNA Mini Kit by Qiagen. Initially, the volume of each sample was adjusted to 250 µL with RNase-free water. Then, 15 µL of Oligotex suspension and 250 µL of OBB buffer, both from the Qiagen kit, were added to the samples. The mixture was heated to 70°C for 3 minutes and subsequently cooled to 25°C for 10 minutes. After centrifuging at 14,000×g for 2 minutes, the supernatants were discarded. The samples were then washed with 400 µL of OW2 wash buffer from the kit and transferred to spin columns provided in the kit, followed by centrifugation at 14,000×g for 1 minute. This washing step was repeated. Finally, the polyadenylated RNA was eluted from the membrane using 50 µl of pre-heated elution buffer from the Qiagen kit, collected in 60 µl elution buffer, with a second elution step performed to maximize the yield (**Supplementary Table S2**).

**RNA quantification**

For measuring total RNA, we used the Qubit RNA BR Assay Kit from Invitrogen (Carlsbad, CA, United States). To quantify the poly(A)+ fraction, the Qubit RNA HS Assay Kit, also from Invitrogen (Carlsbad, CA, United States), was employed. The final concentrations of these RNA samples were determined using the Qubit® 4 fluorometer.

**cDNA quantification and quality assessment**

The concentrations of cDNA samples and sequencing-ready libraries were ascertained using the Qubit dsDNA HS Assay Kit from Invitrogen (Carlsbad, CA, United States). The quality of RNA, crucial for sequencing, was evaluated using the Agilent 2100 Bioanalyzer for PacBio sequencing, and the Agilent 4150 TapeStation System for MinION sequencing. Samples with RIN scores of 9.6 or higher were selected for cDNA synthesis.

**CAGE sequencing**

The Cap analysis of gene expression sequencing (CAGE-Seq) protocol has been previously described [29]. Briefly, using the CAGE™ Preparation Kit (DNAFORM, Japan), we performed CAGE-Seq on viral genomic regions employing three biological replicates. Initially, 5 µg of total RNA and the kit's RT primer were mixed and heated at 65 °C. SuperScript III Reverse Transcriptase (Invitrogen) and a trehalose/sorbitol mixture (from the kit) were used for first-strand cDNA synthesis, followed by oxidation of the Cap's diol groups and biotinylation. RNase I (from the kit) digested single-strand RNA. Biotinylated samples were then bound to Streptavidin beads, washed, and cDNAs were released and purified. RNase mixture treated the samples to digest any residual RNA. Streptavidin beads, coated with tRNA, were prepared for linker ligation. After reducing the sample volumes using the miVac DUO Centrifugal Concentrator (Genevac), 5′- and 3′-linkers were ligated, followed by Shrimp Alkaline Phosphatase (SAP) and USER enzyme treatments. The second cDNA strand was synthesized, treated with Exonuclease I, and samples were dried and resuspended in nuclease-free water. Single-stranded cDNA concentrations were measured using Qubit 2.0 and the Qubit ssDNA HS Assay Kit. Purification steps employed RNAClean XP and AmpureXP Beads at various stages. Pooled libraries with different barcodes were sequenced on a MiSeq instrument using v3 (150 cycles) and v2 (300 cycles) chemistries (Illumina). The final concentration and quality of the libraries were assessed using Qubit 4.0 with a 1X dsDNA High Sensitivity (HS) Assay and TapeStation, respectively.

**Library construction and cDNA sequencing**

Libraries for direct cDNA sequencing on the ONT MinION device were constructed using poly(A)+-enriched samples. We followed the protocol of the ONT Direct cDNA Sequencing Kit (SQK-DCS109), as outlined in the kit's manual. Initially, RNA samples were mixed with VN primer (VNP; from the ONT kit) and 10 mM dNTPs, and heated at 65°C for 5 minutes. This was followed by the addition of 5x RT Buffer, RNaseOUT (from Thermo Fisher Scientific), and Strand-Switching Primer (SSP; from the ONT Kit), and a subsequent 2-minute heating at 42°C. The first cDNA strand synthesis involved the Maxima H Minus Reverse Transcriptase enzyme (from Thermo Fisher Scientific), with the reaction occurring at 42°C for 90 minutes, and enzyme inactivation at 85°C for 5 minutes. RNA strands from RNA-cDNA hybrids were removed using the RNase Cocktail Enzyme Mix (from Thermo Fisher Scientific) at 37°C for 10 minutes. The second cDNA strand was synthesized using LongAmp Taq Master Mix [from New England Biolabs (NEB)] and PR2 Primer (PR2P), with PCR reaction specifics is described in [28]. DNA fragments were then processed for end-repair and dA-tailing using the NEBNext End repair/dA-tailing Module (NEB) at 20°C for 5 minutes, followed by 65°C for 5 minutes. This step was followed by adapter ligation using the NEB Blunt/TA Ligase Master Mix (NEB) at room temperature for 10 minutes. The ONT direct cDNA sequencing (dcDNA-Seq) libraries were barcoded as outlined in [28], and as per the ONT Native Barcoding (12) Kit instructions. The prepared cDNA libraries (200 fmol/flow cell) were purified and loaded onto ONT R9.4.1 SpotON Flow Cells, using a total of five flow cells for sequencing. To prevent "barcode hopping," samples from earlier and later time points were sequenced separately. After each enzymatic step, AMPure XP Beads were used for purification. The samples were then eluted in UltraPure™ nuclease-free water (from Invitrogen), and their concentration was measured using the Qubit 4.0 fluorometer and Qubit dsDNA HS Assay kit.

**Pre-processing**

*dcDNA sequencing*

The raw current signals obtained from ONT-minION sequencing were basecalled to nucleotides with the Dorado-0.7.2 basecaller (<https://github.com/nanoporetech/dorado/>) using a quality threshold of 7. The resulting reads were aligned to the reference genome (accession number: NC\_001491.2) using the minimap2 [30] program. During the alignment with minimap2, the following settings were applied: -ax splice -Y -C5 -cs. To identify TSS, TES, and intron positions, we used the LoRTIA toolkit (<https://github.com/zsolt-balazs/LoRTIA>). For evaluating direct cDNA sequencing (dcDNA-Seq), the following settings were applied in the LoRTIA package: −5 TGCCATTAGGCCGGG –five\_score 16 –check\_in\_soft 15 –3 AAAAAAAAAAAAAAA –three\_score 16 –s Poisson –f true. A transcript was accepted when its 5'- and 3'-adapters were accurate, and in the case of 3'-ends, false priming and template switching during intron identification were excluded. For introns, we accepted those annotated in dRNA sequencing for direct cDNA samples. For further analysis, we used an in-house developed R pipeline. Briefly, the “*stranded\_only.bam*” files from the LoRTIA output were imported into the R environment using Rsmatools [31] . A database was then built from it, containing the count of unique mapping positions and the information from the bam-files regarding LoRTIA’s adapter searching using data.table [32] R-package [33]. This was then used to count the 3'- and 5'-ends per nucleotide and the coverages.

## **Data analysis**

*CAGE-Seq analysis*

The STAR aligner (version 2.7.3 a) [34] was used to map the reads to the EHV-1 reference genome (NC\_001491.2), utilizing --genomeSAindexNbases 8 and default parameters. “Bam” files obtained from CAGE-seq were converted to BigWig format to detect 5′-end coverage. The CAGEfightR [35] package was used to determine TSS positions. The TSS clusters within a 10 nucleotides window were termed identical. Clusters with a “minimum pooled value” (--pooledcutoff=1) of 1 and below were excluded from the further analysis. We assigned confidence levels to TSS clusters derived from the CAGEFighteR output by empirically combining support thresholds with quartile-based score bins. Clusters with support ≤ 2 and scores in the bottom quartile (Q1) were classified as low confidence (labelled with an asterisk [\*]), while those with support of 3–5 and scores in Q2 or Q3 were assigned moderate confidence (labelled with two asterisks [\*\*]). Clusters with support exceeding 5 or scores in the top quartile (Q4) were categorized as high confidence (labelled with three asterisks [\*\*\*]). Clusters meeting only one of the high-confidence criteria were also classified as moderate confidence. This classification is heuristic rather than a formal statistical test, relying on observed distribution patterns to label clusters as low, moderate, or high confidence.

*Reference transcript counting*

The GFF-compare tool [36] was used to count the reference transcripts (from [28]) in the samples. However, since this tool tends to assign shorter transcript isoforms – those contained within another transcript - to the longer one, we ran this tool iteratively for each reference transcript separately. The results were merged, and the best hit for each query alignment was selected based on the smallest distance between transcript ends. For counting reference isoforms, only the hits with “equal to reference” (“=”) were retained, with a distance cut-off of 10 nt-s for both ends. The R-packages rtracklayer [37] was used to export and import .gff3 files.

*TSS validation*

The clusters were merged with the dcDNA-Seq dataset, which contains transcript identities based on the alignment of 5′-ends. TSS clusters were then refined using dcDNA-Seq read counts (with correct 5′-ends), as some clusters spanned up to 150 nts. A custom peak-analysis method was applied to each TSS cluster. Briefly, the algorithm used per-position read counts (summed dcDNA-Seq 5′-ends) as weights and grouped nearby positions into clusters if (i) their genomic distance was at most 5 bp and (ii) the cluster had not already reached its maximum size of 25 positions. Positions with zero coverage were excluded. This process ensured that local “hotspots” of TSS usage were segregated into compact clusters for downstream analyses.

*Transcript assembly and validation*

Transcripts were reconstructed by pairing validated TSS peaks with corresponding TESs using stringent criteria: (i) transcripts were annotated to a novel TSS only if their 5′-ends were within ±10 nts of the refined TSS cluster, and (ii) their 3′-ends overlapped a known TES (also within ±10 nts). This approach enabled the integration of the CAGE-Seq and dcDNA-Seq datasets to annotate TSSs.

To annotate transcripts, we paired the refined TSS peaks with TESs, ensuring that (i) the transcript's 5′-end was within ±10 nts of the refined TSS cluster, and (ii) the corresponding 3′-end overlapped a known TES within ±10 nts. Each transcript required strict criteria for annotation:

● At least three dcDNA-Seq reads sharing the same TSS and TES coordinates,

● Presence of correct 5′-adapter sequences,

● Alignment with CAGE-Seq–derived TSS clusters and previously validated TESs.

Newly assembled transcripts were integrated with our prior annotation [28], allowing us to reintroduce previously excluded transcripts that now met refined criteria, as well as to add novel transcripts not previously detected. Validated transcripts were classified based on their structural and functional features, including coding capacity (e.g., putative mRNAs, non-coding RNAs), monocistronic or multicistronic, and variations in untranslated regions (e.g., shortened or extended isoforms). We further evaluated our previous dRNA-Seq dataset using NAGATA software [38] to validate the novel TSSs and introns identified by LoRTIA. The following settings were applied: -m 1 -tg 2, with all other parameters set to default. This configuration was chosen to annotate rare TSSs, TESs, and introns in the dRNA-Seq data, which were confirmed by the dcDNA-Seq data exhibiting significantly higher read counts.

*Filtering 5′-truncated ORF-carrying transcripts*

To further filter TSS transcripts containing 5′-truncated ORFs with in-frame ATGs (putative mRNAs), the identified TSSs had to meet two criteria: (i) they had to appear in the NAGATA output within a 25 nt wobble range (to account for potentially missing 5′-ends), and (ii) they required validation by CAGE based on their signal strength (5′-end read count) relative to the canonical TSS of their parent gene. Specifically, for a 5′-truncated isoform to be included, its TSS had to exhibit at least 5% expression compared to its canonical transcript.

*Transcript classification*

Transcript isoforms were classified into several groups based on differences in their TSS, TES, and ORF composition compared to the canonical transcript.

* Putative embedded mRNAs: These transcripts contain a 5′-ORF with an in-frame ATG and share a 3′-coterminus with the canonical ORF of the given gene.
* Non-coding RNAs (ncRNAs): transcripts that lack ORFs.
* 5'-UTR isoforms: These can be mono- or polycistronic, containing the same ORF as the canonical transcript but differing in the length of their 5′-untranslated regions (UTRs).
* Antisense RNAs (asRNAs): transcripts antisense to known genes.

*De novo clustering of genes by dynamic expression patterns*

To identify groups of TSSs, TESs, and transcripts with similar temporal expression patterns, we performed *de novo* clustering on normalized gene expression data. For TSS clustering, we used reads from the time-resolved dcDNA-Seq dataset that (i) had a correct 5′-adapter, (ii) aligned with the canonical TSS, and (iii) had the correct 5′-end. For TES clustering, we used reads that (i) had a correct polyA-tail and (ii) whose 3′-ends overlapped with the canonical TES. For gene clustering, only reads fulfilling both criteria were included. Hierarchical clustering was conducted using the pvclust R package [39], which provides hierarchical clustering and bootstrap resampling for cluster validation. The complete linkage method and an uncentered correlation distance measure were applied. Cluster stability and significance were assessed using 1,000 bootstrap iterations, focusing on approximately unbiased (AU) p-values provided by pvclust. After generating a dendrogram, we evaluated cluster solutions ranging from 4 to 15 clusters, assessing their quality based on AU values and within-cluster sum of squares (WSS). Partitioning the data into 12 clusters struck a meaningful balance between resolution and interpretability. The hierarchical tree was cut into 12 clusters, each representing a distinct temporal expression pattern over the time course. Genes with similar temporal expression formed larger clusters, while those with unique kinetic patterns grouped independently.

## **Results**

## **Dynamic EHV-1 transcriptome: general considerations**

In this study, we conducted a comprehensive time-course transcriptomic analysis of EHV-1, primarily using direct cDNA sequencing (dcDNA-Seq) on the ONT MinION platform and incorporating cap analysis of gene expression sequencing (CAGE-Seq) on the Illumina MiSeq platform. Our previous work [28] utilized native RNA sequencing (dRNA-Seq) with stringent filtering criteria, ensuring high confidence but excluding many detected transcripts that failed to meet the strict validation requirements. One key limitation of dRNA-Seq was its reduced accuracy in identifying transcription start sites (TSSs), partly due to the incomplete 5'-ends of the sequencing reads. To overcome these limitations, we integrated CAGE-Seq data with dcDNA-Seq reads. CAGE-Seq provides high-resolution TSS mapping, enabling more accurate identification and validation of 5'-ends. By aligning dcDNA-Seq reads that carried correct 5'-adapters and intact poly(A) tails with CAGE-derived TSS clusters, we confirmed the authenticity of previously excluded transcripts and added 170 novel ones.

For the kinetic analyses, we focused on canonical transcripts - defined as the most abundant isoforms for each viral gene - and their TSSs and TESs. Using these canonical references, we analyzed the temporal dynamics of gene expression across multiple time points, clustered genes into *de novo* kinetic classes based on their expression curves, and compared these classes with the traditional IE/E/L framework.

Finally, beyond annotating full-length transcripts and refining exact TSS/TES positions, we explored isoform switching and transcriptional overlaps, indicating how the virus orchestrates complex regulatory patterns over the infection cycle.

~~In total, this integrated strategy identified and validated 277 additional transcripts and, by examining isoform switching, splicing dynamics, and transcriptional overlaps, significantly enhanced our understanding of the EHV-1 transcriptome and its intricate temporal regulation.~~

**The ORF64 is the only EHV-1 immediate-early** **gene**

It has been previously established that ORF64 is the only immediate-early (IE) gene of EHV-1 [40]. However, our earlier study [28] and the current research have identified novel EHV-1 transcripts with previously unknown kinetic properties. To investigate whether the expression of these transcripts requires newly synthesized viral proteins, we treated RK-13 cells (ECACC: 00021715) with cycloheximide (CHX), a protein synthesis inhibitor, prior to infection with EHV-1. We administered CHX at concentrations of 20 and 100 μg/ml and collected samples at 6 and 8 hours post-infection (hpi). Subsequently, we performed long-read dcDNA sequencing. Our findings reinforced that ORF64 is the sole IE gene in EHV-1 (**Supplementary Table S3**). In pseudorabies virus (PRV), the closest relative of EHV-1 with an annotated transcriptome, the homologous gene (*ie180*) is also the only IE gene [39]. In contrast, other annotated alphaherpesviruses, including those in the Simplexvirus and Varicellovirus genera, have multiple IE genes among their members.

**Reannotation of EHV-1 transcriptome**

In this part of our study, we employed CAGE-Seq on an Illumina MiSeq platform to achieve high-resolution detection of the TSSs of EHV-1 transcripts. The CAGE-Seq data were integrated with dcDNA-Seq reads to validate, refine, and expand our previous transcriptome annotation [28].

Firstly, we validated transcripts previously annotated in our laboratory using dRNA-Seq [28] against the newly acquired CAGE-Seq data (**Supplementary Table S4**). Among the examined transcripts, 251 received the highest level of support (\*\*\*), indicating robust validation, while 47 showed medium support (\*\*) and 51 had the lowest level (\*) (Please refer to Materials and Methods for specifics). Ide definiálni kell, mit jelent ez számszerűleg. Megvan.

Next, we analyzed dcDNA-Seq reads that could not be assigned to previously annotated transcripts due to TSS differences. By aligning the 5′ ends of these reads - validated by correct 5′ adapter sequences (LoRTIA pipeline) - to TSS clusters identified by CAGE-Seq (CAGEfightR), we refined TSS annotations and identified distinct TSS peaks within broad clusters (>200 bp). Final transcripts were constructed by combining these refined TSSs with TESs from our previous annotations. Transcripts were included if they had at least five dcDNA-Seq reads with 5'-ends aligning to validated TSSs from CAGE-Seq. This integrated approach enabled us to recover and validate 136 transcripts previously excluded due to stringent filtering, as well as identify novel transcripts. In total, we validated 169 additional transcripts. These transcripts were categorized as follows: 11 putative embedded genes encoding 3′-coterminal but 5′-truncated transcripts with in-frame ATG; 26 non-coding RNAs (ncRNAs); 80 isoforms of monocistronic transcripts (42 with longer and 38 with shorter UTRs than the canonical transcripts); and 18 isoforms of multicistronic transcripts (8 with longer and 10 with shorter UTRs than the canonical transcripts). Long and short UTR variants share the same ORF as the canonical transcript but differ in the length of their 5′-ends. Putative embedded genes may encode N-terminally truncated proteins. However, due to the phenomenon of cytoplasmic recapping [40], which can produce capped truncated transcripts, and the less robust 5′ support provided by nanopore-based sequencing methods, we applied an additional filtering criterion. Specifically, for a 5′-truncated isoform to be included, its TSS had to be corroborated by dRNA-Seq data and exhibit at least 5% expression (as determined by CAGE-Seq) relative to its canonical transcript. Details of the previously annotated and novel transcripts, including their levels of support based on our validation criteria in the CAGE-Seq and dRNA-Seq data, as well as their counts across the dcDNA-Seq samples, are provided in **Supplementary Table S4**.

**Kinetic characterization of TSSs, TESs, and canonical transcripts**

We next explored the dynamics of TSSs, TESs, and canonical transcripts over the infection cycle and compared these findings with the traditional IE, early (E), and late (L) kinetic classes. The time points associated with IE, E, and L genes are as follows: IE genes are highly expressed at 1–2 hpi, E genes predominate between 2–6 hpi, and L genes are expressed after 6 hpi, with peak expression observed at 8–12 hpi and beyond.

***TSS expression kinetics***

In this part of the study, a temporal expression analysis of the EHV-1 TSSs was conducted (**Figure 1**, **Supplementary Figure S1** and **Supplementary Table S5A**). We obtained that the early genes, such as ORF20, ORF21, ORF30, ORF31, and ORF63 exhibited peak TSS activities as early as 2 hpi, followed by a gradual decline. Conversely, late genes, including ORF11, ORF14, ORF22, and ORF73, began to show substantial TSS activity starting from 4 hpi, reaching their maxima around 8 to 12 hpi. This pattern is consistent with the known function of these genes in either DNA synthesis (E genes), or in producing structural components for virion assembly and egress (L genes). Detailed temporal profiling further elucidated this dynamic landscape by pinpointing specific TSS peak times for individual transcripts. For example, ORF32 showed an early peak at 2 hpi, ORF51 at 6 hpi, and ORF19 at 8 hpi, each followed by a characteristic decline. Additional examples include ORF18, which peaked at 8 hpi, ORF28, which showed a maximum at 6 hpi and then again at 8 hpi, and ORF50, which exhibited peak activity at 4 hpi.

To further clarify these patterns, we performed hierarchical clustering of TSS abundances throughout the infection. This approach grouped genes based on their expression trajectories rather than relying solely on predefined IE/E/L classifications. The clusters largely reinforced the TSS-based kinetics patterns: large clusters often predominantly contained L genes or E genes, reflecting the temporal shifts observed in the individual TSS analyses. For example, clusters dominated by late genes confirmed the existence of a robust late-expression phase, while clusters enriched in early genes validated an early wave of transcription closely following the IE stage. The kinetics of the TSSs grouped according to the *de novo* clustering are shown in **Supplementary Figure S2** and grouped according to traditional kinetic clusters in **Supplementary Figure S3.**

However, the clustering revealed that some genes did not fit neatly into the expected phases. As observed at the single-gene level, several traditionally late genes exhibited earlier-than-anticipated TSS peaks, while some early genes maintained or regained expression at later times. For example, ORF38, typically classified as late, displayed a TSS peak at 6 hpi - more characteristic of early kinetics - while ORF45, also considered late, peaked at 12 hpi and again at 48 hpi. Similarly, ORF54, an early gene, showed a peak at 24 hpi, well beyond the window typically associated with early functions. These "misaligned" genes were placed into mixed clusters containing both early and late markers, suggesting they may belong to transitional or intermediate regulatory states rather than strictly defined classes.

The clustering also highlighted small groups or outliers - single- or double-gene clusters - with unique dynamics patterns that do not align with the canonical IE/E/L framework. These outliers suggest that some genes may follow specialized regulatory circuits, contributing to the intricate temporal orchestration of viral gene expression. Our analysis identified 12 distinct clusters, reflecting the temporal and functional profiles of viral gene expression. Cluster\_12 consists of ORF64, encoding the transcriptional regulator ICP4, underscoring its pivotal role in initiating viral transcription and, interestingly, ORF75 (US8A), traditionally considered late (likely due to detection in one replicate at 1 hpi). Early-dominant TSS clusters, such as Cluster\_1 and Cluster\_3, contain genes like ORF20, ORF21, ORF30, ORF53, and ORF63, involved in nucleotide metabolism and genome replication, peaking early post-infection. Intermediate clusters featuring ORF19, ORF37, ORF55, and ORF76 bridge early and late phases, indicating overlapping or transitional expression profiles. Late-dominant clusters – most notably Cluster\_6 and Cluster\_7 – include genes (e.g., ORF22, ORF24, and ORF42 in Cluster\_6) and (e.g., ORF12, ORF13, and ORF48 in Cluster\_7) that encode proteins involved in virion assembly and packaging, peaking at 8–12 hpi. Overall, these patterns reveal a continuous and overlapping temporal landscape rather than strictly partitioned IE/E/L classes.

***TES expression kinetics***

Our examination of TES dynamics (**Figure 2**, **Supplementary Figure S4** and **Supplementary Table S5B**) reveals a complex and overlapping regulatory landscape, much like what we observed at the TSSs. Many genes align with their expected kinetic classes: E genes such as ORF20, ORF21, ORF30, ORF31, and ORF63 display TES peaks within the first few hours post-infection, while L genes including ORF11, ORF14, ORF22, and ORF73 reach their maxima between 8 and 12 hours post-infection. This overall pattern is consistent with the established roles of E genes in DNA replication and L genes in virion assembly. However, both the initial analysis of individual TES kinetics and the subsequent clustering based on TES usage reveal exceptions and overlapping dynamics that challenge the straightforward IE/E/L model. For example, ORF32 and ORF51, traditionally classified as late, exhibited earlier-than-expected TES peaks, while ORF19, categorized as early, showed a delayed TES maximum more characteristic of L genes. These anomalies suggest that the timing of transcript termination does not always correspond to the canonical temporal classes. However, an important reason for this anomaly is that tandem gene clusters produce co-terminal transcripts with distinct temporal expression profiles, which blur IE/E/L boundaries.

The clustering of TES expression profiles (**Supplementary Figures S5 and S6**) revealed distinct groups of genes with shared termination dynamics. For example, clusters predominantly composed of late-expressed structural and assembly genes - such as ORF12, ORF13, and ORF14 in Cluster\_6 or ORF15-18 in Cluster\_8 - highlight the coordinated late-phase production of capsid, tegument, and packaging proteins. In contrast, clusters enriched in early genes - such as ORF7, ORF30, and ORF63 in Cluster\_5 - peak during the initial stages of infection, consistent with their roles in replication and regulation.

Mixed-phase clusters are particularly insightful. Sets such as ORF32–34 in Cluster\_7 or ORF48–51 in Cluster\_3 combine genes from both early and late classes, indicating that replication factors and structural proteins can share the same termination site. Again, this co-termination creates overlapping kinetic patterns, emphasizing a continuous temporal landscape rather than strictly segmented IE/E/L phases.

Smaller clusters also provide meaningful insights. For instance, Cluster\_11, which includes ORF64, underscores its unique regulatory role at the TES level. Other clusters, such as those containing envelope glycoproteins and tegument proteins in Cluster\_10 or multi-gene E/L sets in Cluster\_1 and Cluster\_12, illustrate that transcripts with differing temporal classes can terminate together, further increasing transcriptional complexity.

***Aligning TSSs with TESs***

To investigate the similarities and discrepancies between TSS and TES kinetics, we performed a detailed analysis of the linkage between TSS and TES sites using our lrRNA-Seq data. By matching TSSs to TESs on individual transcript molecules, we were able to assess whether the observed differences in kinetics were due to the presence of alternative transcript isoforms, multicistronic transcripts, or other factors. **Figure 3** shows each gene's abundance during the course of the infection, as assessed by the viral-read-normalized canonical transcript counts, according to their kinetic classes.

For genes where TSS and TES dynamics differed, our analysis revealed that the discrepancies could often be attributed to the complex transcriptional landscape of EHV-1. The virus produces a variety of transcript isoforms, including alternative TSSs and sometimes TESs, as well as multicistronic and overlapping transcripts **(Figure 4, Supplementary Figure S7** and **Supplementary Table S5C**). For example, both ORF38 and ORF50, expected to show late kinetics, exhibited early peaks, although their TSS peaked at 4 and 8 hpi, while their TES at 6 hpi. This misalignment is in the case of ORF38 the result of the elevated expression of other, mainly complex transcripts (overlapping ORF35-37 from the other strand) that terminate at the same TES, such as ORF37-ORF38-CX-Long-2 (**Figure 4A)** and possible transcriptional noise from other non-validated TSSs in this region. In the case of ORF50 (**Supplementary Figure S7B**), this could be attributed to a more complex differential transcript expression pattern consisting of mainly ORF50-ORF51-Canonic and ORF50-ORF51-PC-Long-2. Conversely, ORF67, which showed an early TSS peak, had TES dynamics more consistent with its L gene classification (**Supplementary Figure S7C**). While its presence at 1 hpi could be attributed to transcriptional noise affecting this very early time point, at 2 hpi it is more likely due to the highly efficient early activation of its promoter. The discrepancy between its TSS and TES kinetics can be explained by the large number of potential mRNAs identified in this region, each carrying 5′-truncated ORFs, albeit with individually low counts. Our detailed mapping confirms that the discrepancies between TSS and TES kinetics are primarily due to the production of multiple transcript isoforms and the complex arrangement of transcription units in the EHV-1 genome. This underscores the importance of examining full-length transcript structures when interpreting gene expression dynamics, a task for which LRS techniques are the only reliable approach.

***Gene-level clustering of canonical transcripts***

Clustering canonical full-length transcripts provides a cleaner view of EHV-1’s transcriptional program. **Figure 5** shows the genes sorted into *de novo* kinetic clusters.

*Mixed clusters:*

Cluster\_1 contains a mix of E genes (e.g., ORF20, ORF21, ORF31, ORF61), L genes (e.g., ORF9, ORF38, ORF50), and genes with undetermined kinetics. It peaks around 2–4 hpi, indicating "leaky-late" activity within an early-expression context. Similarly, Cluster\_3 is early-biased (e.g., ORF5, ORF7, ORF30, ORF53, ORF63) but includes a few L genes (e.g., ORF10, ORF17). This pattern reinforces the idea that some L transcripts are detectable at low levels early on, blending replication and assembly factors in a transitional manner between 2–4 hpi, possibly as mere transcriptional noise.

*Robust late-dominant clusters:*

Cluster\_2 predominantly comprises L genes (e.g., ORF11, ORF14, ORF18, ORF26, ORF28, ORF29, ORF3, ORF39, ORF40, ORF68, ORF73, ORF76) along with a few genes of undetermined kinetics (e.g., ORF2, ORF75). This cluster aligns with a robust late-phase expression wave emerging after 6–8 hpi. Similarly, Cluster\_5 consists of late structural and packaging components (e.g., ORF22, ORF23, ORF25, ORF33, ORF35.5, ORF36, ORF42, ORF43, ORF44, ORF46, ORF58, ORF62), which steadily produce virion-related proteins during mid-to-late infection.

Clusters\_6 and \_7 are also late-dominated but include notable exceptions. For instance, Cluster\_6 primarily consists of late genes (e.g., ORF12, ORF13, ORF16, ORF35, ORF41, ORF48, ORF52, ORF57, ORF60, ORF72) involved in tegument formation and packaging. However, it also includes one early gene (ORF54) and one of undetermined kinetics (ORF71), reflecting the complexity of late-phase expression. Similarly, Cluster\_7 combines predominantly late genes with overlapping temporal profiles.

*Special clusters: mitől más ez, mit az első klaszter, ami szintén mix?Itt kisebb 1-2 gént tartlmazó klaszterek vannak (a másikban sok gén van), erre vonatkozik hogy special, tehát kvázi gén-specifikus, de lehet máshogy is megnevezni*

Cluster\_4 and Cluster\_12 contain fewer genes and exhibit mixed kinetics. Cluster\_4 includes both L (e.g., ORF6, ORF67) and E genes (e.g., ORF65), indicating subtle overlaps even in smaller sets. Meanwhile, Clusters\_8, \_9, and \_10 refine subsets of late genes or highlight unique outliers. Notably, Cluster\_10 contains the sole IE gene, ORF64. Although no canonical full-length transcripts were detected at 1 hpi - likely due to technical challenges capturing this very long RNA - the clear isolation of ORF64 within its own cluster underscores its distinct temporal regulation (**Supplementary Figure S7D**).

Collectively, these clusters confirm that while the IE/E/L scheme provides a broad framework, actual gene expression patterns form a continuous and overlapping temporal gradient. **Supplementary Figure S8** shows the *de novo* cluster memberships of each gene, along with their kinetic classes. By integrating these analyses, we observe a consistent narrative: while many EHV-1 genes follow the classical IE, E, and L progression, a subset displays more complex or hybrid kinetics. Gene-level TSS analysis highlights individual timing anomalies, while clustering places these anomalies in a broader context, revealing that they form part of overlapping transcriptional waves rather than discrete phases. These findings suggest that EHV-1 gene regulation is multifaceted, with certain genes bridging temporal classes and potentially serving specialized regulatory or structural roles at unconventional times during infection.

**Dynamics of transcriptional overlaps and transcript isoforms**

***Dynamics of spliced transcript expression***

The splice sites of EHV-1 transcripts were previously identified in our laboratory using native RNA sequencing. We detected splice sites in the following genes: ORF8, ORF9, ORF38, ORF47-44, ORF53, ORF54, ORF58, ORF65, and within the NOIR family of non-coding transcripts. **Figure 4** and **Supplementary Figure S9** illustrate these genes. For several of them, we observed notable shifts in the ratio of spliced to non-spliced transcripts over the course of the infection.

In the gene ORF9, the combined ratio of spliced transcripts TR134 and TR172 - both sharing an intron and carrying a 5′-truncated ORF with in-frame ATG - remained at 0% (mean = 0.0) from 1–8 hpi (**Figure 4C**). Their ratio began to rise at 12 hpi (mean = 1.03%, SD = 0.84%), increased significantly at 18 hpi (mean = 11.49%, SD = 5.65%), continued to increase at 24 hpi (mean = 12.85%, SD = 3.60%), and peaked at 48 hpi (mean = 17.10%, SD = 3.64%). Other non-spliced isoforms also elevated compared to the canonical ORF9 transcript, which dropped from 100% early at 2–4 hpi with 100% expression (mean = 100.0%), to only 41.69% (SD = 10.30%) by 48 hpi.

In the case of ORF38 (**Figure 4A**), we saw a very similar pattern, albeit the canonical transcript decreased in proportion even more, to 26.36% at 24 hpi and 27.70% at 48 hpi, reflecting a sharper decline compared to ORF9. This decrease in the canonical transcript was accompanied by a marked increase in the ratios of spliced transcripts TR886, TR888, TR889, and TR891, whose combined ratios rose significantly at 24 hpi and remained elevated at 48 hpi. These spliced transcripts share a second exon (which carries the ORF) and an identical intron but differ in their first exons, which define distinct 5′-UTRs. This suggests that the splicing process is tightly regulated, driving transcript diversity and contributing to the sharp decline of the canonical transcript. Fent azt írod, hogy a spliced verzió változik nem-spliceolttá, itt meg fordítva!!! Ezt nem értem, az ORF 38 esetében a splice-olt (és más izoformák is) nőnek meg a kanonikus (nem splice-olt) pedig csökken, és ez is van leírva illetve a Fig4A-n is ez látszik.

The spliced transcripts TR148, TR150, TR152, TR154, and TR3 of ORF65 showed a common expression pattern. Their combined ratio peaked early at 2 hpi with 66.59%, remained high at 4 hpi (66.37%) and 8 hpi (59.76%), declined to 40.63% by 6 hpi, and decreased further through 12 hpi (59.05%), 18 hpi (15.97%), 24 hpi (17.22%), and 48 hpi (20.01%), reflecting their predominant expression during the early stages of infection (**Supplementary Figure S9A**). These spliced transcripts share the second exon, which carries the ORF, but differ in their intron donor sites or the 5′-ends of their first exons.

The ORF44 transcriptional landscape features a mix of non-spliced and spliced isoforms, with distinct temporal dynamics and TSS validation patterns. The canonical transcript and short isoform (ORF44-S) are non-spliced and fully supported by CAGE, peaking transiently at 8 hpi. Among spliced isoforms, TR416 and TR418 lack CAGE support for their TSS but were retained due to validation in a prior transcript collection; they show early peaks at 12 hpi (11.26%) and 6 hpi (77.78%), respectively. Conversely, TR419, supported by CAGE, shares the same second exon and intron as TR416 and TR418 but features a distinct TSS, peaking at 12 hpi (25.74%). Robust transcripts like TR407 and TR412 exhibit sustained mid-to-late expression, with peaks at 8 hpi (25.40%) and 24 hpi (18.98%), respectively. This highlights the importance of integrating complementary datasets to capture the full complexity of transcriptional regulation in ORF44.

NOIR, a novel non-coding, replication origin-associated RNA (raRNA) has recently been described [28]. The canonical NOIR transcripts (NOIR-canonical and NOIR-ORF65-PC-canonical) showed higher expression at all time points, peaking at 12 hpi (46.08% vs. 3.79% for isoforms), while the splice isoforms contribute modestly throughout but increase relatively at later stages, reaching 14.98% at 24 hpi and 18.46% at 48 hpi compared to 35.02% and 31.15% for the canonical transcripts (**Figure 4D**). Similar to other genes, the spliced NOIR transcripts share a second exon and an identical intron acceptor site but differ in their intron donor sites and/or TSS. In ORF58 (**Supplementary Figure S9D**), the canonical ORF58 transcript peaked at 6 hpi (96.30%) and declined steadily thereafter. ORF58-L1 exhibited an early peak at 2 hpi (33.33%) before decreasing to negligible levels. The spliced transcript TR1072 showed negligible expression early on but increased significantly at 24 hpi (16.54%) and remained prominent at 48 hpi (15.12%), highlighting distinct temporal expression patterns within this gene.

***Dynamics of transcriptional overlaps and isoform switching in selected genes***

To gain a deeper understanding of the complexity of the transcriptional patterns in EHV-1, we analysed the kinetics of transcriptional overlaps and the relative abundance of transcript isoforms of genes. By examining changes in isoform dominance over time, we uncovered dynamic patterns that highlight how the virus fine-tunes gene expression at various stages of infection. In **Figure 6**, we illustrate these transcripts and their shifting proportions, revealing that even canonical transcripts can be temporarily superseded by truncated or alternatively terminated isoforms, reflecting a finely controlled temporal program of viral gene expression. **Figure 7** and **Supplementary Figure S10** highlights the significant amount of convergent and divergent transcriptional overlaps in EHV-1, increasing as the infection proceeds.

The expression dynamics of ORF19 revealed a transition from early dominance of the canonical transcript, which peaked at 1 hpi (100%) and declined to 14.59% by 48 hpi, to increasing contributions from the combined complex isoforms (CX), overlapping completely the CDS of ORF18 on the other strand, which rose from negligible levels at 1–4 hpi to 41.44% at 12 hpi and peaked at 72.21% at 48 hpi (**Figure 6A**).

The canonical ORF40 transcript dominated expression during the early stages, peaking at 6 hpi (97.78%) before declining sharply. Alternatively terminated (AT) isoforms contributed minimally during early stages but increased in abundance later, with AT2 reaching a peak of 37.42% at 24 hpi, while other AT isoforms remained relatively low in expression throughout the time course (**Figure 6B**).

The canonical transcript of ORF13 (ORF13-ORF14-PC-Canonic) showed increasing expression from 2 hpi (8.33%) to a peak at 48 hpi (73.45%), while the long isoform (ORF13-ORF14-PC-Long) dominated early expression, peaking at 4 hpi (100%) and gradually declining to 26.22% at 48 hpi (**Figure 6C**).

In ORF14, transcript dynamics revealed distinct patterns and isoform switching among the canonical isoform, the long variants (L), and the truncated variant (ORF14.5, representing a 5′-truncated transcript). The canonical isoform dominated early expression, peaking at 63.89% at 2 hpi, and did not regain prominence later, showing a steady decline. The long variants (L) contributed modestly, with specific peaks such as L4 at 18 hpi (3.89%), while others like L1 and L3 remained low throughout, peaking below 1% by 48 hpi. The truncated variant ORF14.5 dominated the mid-phase, peaking at 78.54% at 6 hpi and remaining the most abundant transcript until 18 hpi, before declining to 24.44% at 48 hpi (**Supplementary Figure S9E**).

In the gene ORF11, the canonical ORF11 transcript peaked at 8 hpi (86.65%) before declining, while its 5′-truncated ORF-carrying ORF11.5 isoform showed an inverse pattern, peaking early at 4 hpi (74.72%) and decreasing steadily thereafter (**Supplementary Figure S9F**).

For ORF54, the short (S) isoform dominated early expression, peaking at 2 hpi (100%) and declining sharply after 6 hpi, while the canonical transcript emerged later, surpassing the short isoform at 8 hpi (48.85% vs. 22.47%) and dominating mid-to-late stages, peaking at 18 hpi (96.36%) and maintaining high levels through 48 hpi (93.17%). (**Supplementary Figure S9C**). Nem sorban vannak említve a panelek, és van ami nincs említve!!! A supplementaryk esetében bármilyen sorrendben lehet hivatkozni a panelekre, ez külön le van írva a plos one-ban. És mivel annyi varia volt a figure-kkel, hogy ezt végül így hagytam. Ez nem hiba, de ha szeretnéd meg lehet cserélni.

**Comparison of replication origin-associated transcripts of EHV-1 and PRV**

## The raRNAs are mapped near the replication origins (Oris) in herpesvirus genomes. The NOIR ncRNA has been described to map between the ORF64 and ORF65 transactivator genes near the OriS [28]. A similar ncRNA was also detected in PRV, where it resides between the homologous IE180 and US1 genes. Compared to our previous publication [29], CAGE-Seq analysis enabled us to update the other raRNA, CTO-S, located at Ori-L (**Supplementary Figure S11A**). CTO has only been described in EHV-1 and PRV (**Supplementary Figure S11B**), while NOIR has been identified in Varicelloviruses [11]. Since these are ncRNAs, their level of conservation is lower than that of the protein-coding regions.

## **Discussion**

## The last couple of years have witnessed significant advancements in sequencing technologies [41]. Full-length, lrRNA-Seq methods have revolutionized transcriptome research, particularly in organisms with small genomes. This has revealed that viral transcriptomic structures are far more complex than previously thought [11]. Discoveries include a wide array of overlapping transcripts, such as extended 5′-UTR isoforms, polygenic and complex transcripts (containing at least one gene on an opposite orientation), 5′-truncated mRNAs containing in-frame ORFs, and read-through transcripts [21,42–44]. Recent studies have demonstrated that the occurrence of nested genes within a larger canonical gene is more prevalent in viruses than previously believed [23,44,45]. Neither SRS nor LRS techniques alone are effective in detecting the products of these nested genes. This insufficiency stems from the tendency of lrRNA-Seq techniques to cause considerable 5'-truncation in transcripts, potentially leading to the misidentification of TSSs [46]. To address this issue, we utilized CAGE sequencing via the Illumina MiSeq platform, a standard approach for identifying the 5'-ends of capped RNA molecules. Although CAGE-Seq is generally reliable, it has the potential for detecting some fraction of degraded RNA. This possibility arises because, in mammalian cytoplasm degraded RNAs can be capped by special host enzymes [47]. Although these incomplete transcripts originate from biological processes and may be functional, we attempted to minimize this form of 'noise' by setting a high score threshold for recognizing them as true TSSs produced through transcription.

## Polycistronism is a common feature in bacteria and viruses, but it is rare in eukaryotes. In prokaryotes and bacteriophages, a ribosomal binding site on the mRNAs, known as the Shine-Dalgarno sequence, facilitates the translation of downstream genes in polycistronic RNA molecules. Many small-genome eukaryotic viruses have evolved various mechanisms, such as internal ribosome entry sites, ribosomal frameshifting, or leaky ribosomal scanning [48]. In herpesviruses, co-oriented genes often form clusters that produce transcripts with shared downstream sequences and unique 5′-exons, following a pattern like ‘abcd’, ‘bcd’, ‘cd’, and ‘d’, where ‘a’ is the most upstream gene and ‘d’ is the most downstream. The role of polygenic transcripts in large DNA viruses remains unclear, as translation from downstream genes has been rarely documented [49,50].

We have previously reported that EHV-1 exhibits more frequent splicing events compared to related alphaherpesviruses [28]. Transcripts of ORF44 (homolog of HSV ul15), ORF65 (homolog of HSV us1), and NOIR are spliced in other alphaherpesviruses as well. However, EHV-1 uniquely features splicing in different genomic regions, such as ORF6/12, ORF35/39, and ORF53/58. Additionally, the splicing observed in ORF44 extends to adjacent genomic areas, including ORF49/50, which is a distinctive characteristic of this virus.

An ongoing challenge in long-read RNA sequencing studies is that most existing pipelines prioritize the most abundant isoforms and rely on reference annotations primarily designed for eukaryotic transcriptomes, often overlooking the complexities typical of viral genomes. Recent benchmarks, for instance, have focused on synthetic or mammalian datasets without addressing the extensive overlap, alternative TSSs, and co-terminal transcripts that frequently occur in viruses [51]. Tools such as StringTie2 and gff-compare, while effective for annotating standard eukaryotic transcripts, often collapse shorter isoforms into a single long isoform. This issue is exacerbated in viruses, where numerous potential TSSs and overlapping ORFs create a dense genomic landscape. NAGATA, a pipeline reportedly applicable to viruses, has shown promise for native RNA sequencing data [38]. However, it explicitly discards 5′ soft-clipped reads, making it unsuitable for dcDNA-Seq libraries that rely on 5′-adapter sequences to orient alignments. In contrast, our LoRTIA pipeline accommodates these 5′-adapter sequences, using them to determine strand information and effectively process dcDNA-Seq data. Nevertheless, LoRTIA also faces the fundamental challenge of distinguishing genuine 5′-truncated transcripts from artifacts introduced by incomplete reverse transcription or cytoplasmic mRNA recapping. The latter can generate novel 5′-ends that may be biologically meaningful [40], but these risk being misidentified as legitimate viral TSSs without careful filtering.

In view of these constraints, our study focused on capturing canonical full-length viral transcripts while also monitoring alternative TSSs and TESs at lower abundance. By integrating multiple data sources (dcDNA-Seq, dRNA-Seq, and CAGE-Seq) and using different tools - including LoRTIA for dcDNA-Seq libraries and NAGATA for dRNA-Seq data - we applied stringent criteria, particularly for 5′-truncated transcripts, to minimize false positives without overlooking potentially functional low-abundance isoforms. Additionally, we performed a peak analysis on TSS clusters identified from the CAGE data (CAGEfightR), refining 5′-boundaries using dcDNA-Seq read counts. Together, these measures provide a robust, flexible strategy for accurately annotating viral transcripts under current methodological and computational constraints.

In this work, we integrated dcDNA-Seq and CAGE-Seq data to create a comprehensive, time-resolved map of the EHV-1 transcriptome. By leveraging the strengths of each method - CAGE-Seq for high-resolution TSS identification and dcDNA-Seq for capturing full-length transcripts - we validated previously excluded transcripts and identified a substantial number of new isoforms. We refined TSS and TES annotations, discovered multiple spliced variants, and documented dynamic patterns of isoform switching, highlighting the EHV-1 transcriptome's complexity and fluidity beyond previous understanding. This integrated approach also enabled us to cluster genes into *de novo* kinetic classes, revealing overlapping temporal waves of expression that transcend the traditional IE/E/L framework.

The implications of our findings are significant for understanding the regulatory strategies of EHV-1. The presence of numerous transcript isoforms and intricate splicing dynamics suggests that the virus employs multiple layers of post-transcriptional control. Temporal shifts in isoform prevalence, along with alternative TSSs and TESs, indicate that EHV-1 fine-tunes gene expression throughout the infection cycle. The complexity arising from the vast number of transcript isoforms likely enables the virus to respond flexibly to host conditions, optimize resource utilization, and orchestrate the production of viral components for efficient replication and spread.

We believe that the significance of these isoforms arises not only from their coding capacity but also from their ability to physically inhibit the transcription of other genes through the process of transcriptional interference[11]. For instance, the early increase in the 5'-truncated isoform of **ORF11** could indicate a regulatory mechanism to quickly produce a necessary protein without the full-length transcript, while the later increase may reflect a need for the complete protein function in later stages. On the other hand, the steady increase in **ORF40** AT isoforms (TES variants) suggests a role in interfering with the transcription initiation of its adjacent gene, the CTO, which is the most abundant non-coding transcripts.

Taken together, our results emphasize that EHV-1 gene regulation is governed by a sophisticated and multi-layered transcriptional program. By delineating the full complement of viral transcripts and their temporal patterns - including splicing and isoform switching - we provide a solid foundation for future studies to unravel the molecular mechanisms underlying viral replication and pathogenesis.

## **Data availability:**

The sequencing datasets generated in this study are available at the European Nucleotide Archive under the accession: PRJEB52190 and PRJEB6233.

The R codes used to perform the analysis and generate the plots are available at: <https://github.com/Balays/EHV-1-dynamic>

**Ethics statement**

Ethics approval is "Not Applicable," as no animal experiments were performed.

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## **Legends to Figures**

**Figure 1. Kinetics of transcription start sites of EHV-1 detected by dcDNA-Seq and validated by CAGE-Seq.**

The time-course experiment utilizing dcDNA-Seq spanned 8 timepoints, ranging from 1 hour to 24 hours. TSSs were identified through LRS analysis and confirmed via CAGE-Seq. For each nucleotide, we counted the number of reads beginning at that position with their 5'-ends. We included only those reads that had clear directionality, which was determined by the presence of 5'-or 3'-adapters. Data from all three replicates were combined.

(a) We then grouped the TSS signal strength values into 50-nt segments to illustrate the distribution of TSSs. The y-axis of the graph was automatically scaled to accommodate up to 500 read counts. An image with lower (5,000 read counts) and higher (50 read counts) resolution details can be found in Supplementary Figure 1a and 1b, respectively. In the representation, genes are indicated by arrows, and the distribution of TSSs is shown in different color: red for the positive strand and blue for the negative strand. The bottom row of the image displays the CAGE-Seq counts.

(b)To illustrate the distribution of TSSs within each group, we normalized the signal strength by dividing it with the total signal strength observed for all samples in the same hour post-infection group (and the CAGE-Seq). This approach allows for a comparative analysis of TSS distribution relative to the group's overall viral read count.

**Figure 2. Kinetics of transcription end sites of EHV-1 detected by dcDNA-Seq and validated by dRNA-Seq.**

The time-course study covered 8 intervals, from 1 to 24 hours. The TESs were detected using oligo(dT) priming-based dcDNA sequencing, which subsequently were confirmed with dRNA-Seq. We counted the reads initiating from each nucleotide position with their 3'-ends, focusing only on those with clear directional cues identified by the presence of 5'- or 3'-adapters. Data from all three dcDNA-Seq replicates were merged.

(a) The aggregated read counts were then summed into 50-nt blocks to illustrate the TES distributions. The graph's y-axis was set to automatically adjust, supporting up to 500 read counts. Images offering lower (5,000 read counts) and higher (50 read counts) resolution views are available in Supplementary Figures 4a and 4b. The diagrams mark genes with arrows and color-codes the TSS distribution, using red for the positive strand and blue for the negative strand.

(b)To illustrate the distribution of TESs across each group, we adjusted the read counts at each position (prior to aggregating them into blocks) by the overall read count for all samples in the same time interval group. This approach enables a comparison of TES distribution against the total viral read count for the group.

**Figure 3. Kinetic profiling of canonical EHV-1 transcripts using total viral read counts for normalization according to kinetic classes**

This figure illustrates the kinetic profiling of canonical EHV-1 transcripts, utilizing the total viral read counts per sample for normalization. The analysis included only those reads that aligned with both the canonical TSS of genes at their 5'-ends and the canonical TES of genes at their 3'-ends (allowing a deviation of +/- 10 nt for both alignments). This method aggregated the counts of canonical transcripts for each gene in every sample. The mean values are represented as points, and standard deviations (SD) as lines, plotted on the y-axis as the ratio of transcript abundance for each gene. The x-axis represents time post-infection (hours). The panels are color-coded based on kinetic transcription phases: blue for immediate early (IE), orange for early (E), green for late (L), and red for unknown phases. This provides a visual distinction among different transcriptional dynamics throughout the infection.

**Figure 4. Dynamics of transcript isoform usage in splice-containing EHV-1 genes over the course of infection**

This figure presents the splicing dynamics within EHV-1 for the genes (a) ORF38, (b) ORF8, (c) ORF9, (d) NOIR, (e) ORF58 and (f) ORF53. The right side of each panel shows the transcript annotations, along with their parent genes and genomic locations displayed below them, with light red indicating positive-strand genes and light blue indicating negative-strand genes. The analysis focused on transcripts that matched exactly, allowing a deviation of +/- 2 nucleotides (nt) for splice junctions and +/- 10 nt for the start and end positions of transcripts. Asterisks indicate the CAGE-Seq significance level for each reference transcript. On the left side of each plot, the temporal trends of these transcript isoforms are depicted, with averages and standard deviations (SD) calculated for each time point post-infection, based on read count data from the dcDNA-Seq. Each data point is linked by lines to demonstrate the progression over time. The transcript isoforms, are color-coded according to their distinct isoforms, with these color matching those used for the points and lines in the left panel. The isoform counts were normalized against the total number of isoform counts for each gene in each sample to calculate the ratio of each isoform. Isoforms on the right side are colored grey, if they not originate from the given gene and thus were not included in the isoform ratio calculation.

**Figure 5. Kinetic profiling of canonical EHV-1 transcripts using total viral read counts for normalization according to *de novo* kinetic clusters**

This figure illustrates the kinetic profiling of canonical EHV-1 transcripts, utilizing the total viral read counts per sample for normalization. The analysis included only those reads that aligned with both the canonical TSS of genes at their 5'-ends and the canonical TES of genes at their 3'-ends (allowing a deviation of +/- 10 nt for both alignments). This method aggregated the counts of canonical transcripts for each gene in every sample. The mean values are represented as points, and standard deviations (SD) as lines, plotted on the y-axis as the ratio of transcript abundance for each gene. The x-axis represents time post-infection (hours). Each cluster is colored according to its *de novo* kinetic cluster membership. The color-coding for the clustering is shown in the bottom right panel. This figure provides a visual distinction among different transcriptional dynamics, according to the gene’s relative abundance throughout the infection.

**Figure 6. Kinetics of transcript variants for isoform switching genes**

This figure illustrates the dynamics of different transcript isoforms for the selected EHV-1 genes (a) ORF11, (b) ORF19, (c) ORF13, (d) ORF40, (e) ORF14, and (f) ORF54.

The right side of each panel shows the transcript annotations, along with their parent genes and genomic locations displayed below them, with light red indicating positive-strand genes and light blue indicating negative-strand genes. The analysis focused on transcripts that matched exactly, allowing a deviation of +/- 2 nucleotides (nt) for splice junctions and +/- 10 nt for the start and end positions of transcripts. Asterisks indicate the CAGE-Seq significance level for each reference transcript. On the left side of each plot, the temporal trends of these transcript isoforms are depicted, with averages and standard deviations (SD) calculated for each time point post-infection, based on read count data from the dcDNA-Seq. Each data point is linked by lines to demonstrate the progression over time. The transcript isoforms, are color-coded according to their distinct isoforms, with these color matching those used for the points and lines in the left panel. The isoform counts were normalized against the total number of isoform counts for each gene in each sample to calculate the ratio of each isoform. Isoforms on the right side are colored grey, if they not originate from the given gene and thus were not included in the isoform ratio calculation.

## **Legends to Supplementary Figures**

**Supplementary Figure 1. Kinetics of transcription start sites of EHV-1 detected by dcDNA-Seq and validated by CAGE-Seq.**

(a) *5,000 limit***.** Similar to Figure 1, this plot shows the 5'-end distribution along the viral genome for each time-point group. The mean values for each time-point group were calculated and merged into 50-nt sized bins for visualization. The y-axis was limited to 5,000 counts.

(b) *50 limit***.** Similar to Figure 1, this plot shows the 5'-end distribution along the viral genome in each time-point groups. The mean values for each time-point group was calculated and merged into 50-nt sized bins for the visualization. The y-axis was limited to 5,000 counts.

**Supplementary Figure 2. Kinetic profiling of canonical EHV-1 TSSs according to *de novo* kinetic clusters**

This figure illustrates the kinetic profiling of canonical EHV-1 TSSs, utilizing the total viral read counts per sample for normalization. The analysis included only those reads that aligned with the canonical TSS of genes at their 5'-ends (allowing a deviation of +/- 10 nt). The mean values are represented as points, and standard deviations (SD) as lines, plotted on the y-axis as the ratio of TSS abundance for each gene. The x-axis represents time post-infection (hours). Each cluster is colored according to its *de novo* kinetic cluster membership. The color-coding for the clustering is shown in the bottom right panel. This figure provides a visual distinction among different transcriptional dynamics, according to the gene’s relative TSS abundance throughout the infection.

**Supplementary Figure 3. Kinetic profiling of canonical EHV-1 TSSs according to kinetic classes**

This figure illustrates the kinetic profiling of canonical EHV-1 TSSs, utilizing the total viral read counts per sample for normalization. The analysis included only those reads that aligned with the canonical TSS of genes at their 5'-ends (allowing a deviation of +/- 10 nt). The mean values are represented as points, and standard deviations (SD) as lines, plotted on the y-axis as the ratio of TSS abundance for each gene. The x-axis represents time post-infection (hours). The panels are color-coded based on kinetic transcription phases: blue for immediate early (IE), orange for early (E), green for late (L), and red for unknown phases. This provides a visual distinction among different TSS dynamics throughout the infection.

**Supplementary Figure 4. Kinetics of transcription end sites of EHV-1 detected by dcDNA-Seq and validated by dRNA-Seq.**

(a) *5,000 limit.* Similar to Figure 2, this plot shows the 5'-end distribution along the viral genome in each time-point groups. The mean values for each time-point group was calculated and merged into 50-nt sized bins for the visualization. The y-axis was limited to 50 counts.

(b) *50 limit.*Similar to Figure 2, this plot shows the 5'-end distribution along the viral genome in each time-point groups. The mean values for each time-point group was calculated and merged into 50-nt sized bins for the visualization. The y-axis was limited to 50 counts.

**Supplementary Figure 5. Kinetic profiling of canonical EHV-1 TESs according to *de novo* kinetic clusters**

This figure illustrates the kinetic profiling of canonical EHV-1 TESs, utilizing the total viral read counts per sample for normalization. The analysis included only those reads that aligned with the canonical TSS of genes at their 5'-ends (allowing a deviation of +/- 10 nt). The mean values are represented as points, and standard deviations (SD) as lines, plotted on the y-axis as the ratio of TES abundance for each gene. The x-axis represents time post-infection (hours). Each cluster is colored according to its *de novo* kinetic cluster membership. The color-coding for the clustering is shown in the bottom right panel. This figure provides a visual distinction among different transcriptional dynamics, according to the gene’s relative TES abundance throughout the infection.

**Supplementary Figure 6. Kinetic profiling of canonical EHV-1 TESs According to kinetic classes**

This figure illustrates the kinetic profiling of canonical EHV-1 TESs, utilizing the total viral read counts per sample for normalization. The analysis included only those reads that aligned with the canonical TES of genes at their 5'-ends (allowing a deviation of +/- 10 nt). The mean values are represented as points, and standard deviations (SD) as lines, plotted on the y-axis as the ratio of TES abundance for each gene. The x-axis represents time post-infection (hours). The panels are color-coded based on kinetic transcription phases: blue for immediate early (IE), orange for early (E), green for late (L), and red for unknown phases. This provides a visual distinction among different TSS dynamics throughout the infection.

**Supplementary Figure 7. Kinetics of transcript isoforms for selected genes**

This figure illustrates the dynamics of different transcript isoforms for the selected EHV-1 genes (a) ORF23, (b) ORF51, (c) ORF67, (d) ORF64.

The right side of each panel shows the transcript annotations, along with their parent genes and genomic locations displayed below them, with light red indicating positive-strand genes and light blue indicating negative-strand genes. The analysis focused on transcripts that matched exactly, allowing a deviation of +/- 2 nucleotides (nt) for splice junctions and +/- 10 nt for the start and end positions of transcripts. Asterisks indicate the CAGE-Seq significance level for each reference transcript. On the left side of each plot, the temporal trends of these transcript isoforms are depicted, with averages and standard deviations (SD) calculated for each time point post-infection, based on read count data from the dcDNA-Seq. Each data point is linked by lines to demonstrate the progression over time. The transcript isoforms, are color-coded according to their distinct isoforms, with these color matching those used for the points and lines in the left panel. The isoform counts were normalized against the total number of isoform counts for each gene in each sample to calculate the ratio of each isoform. Isoforms on the right side are colored grey, if they not originate from the given gene and thus were not included in the isoform ratio calculation.

**Supplementary Figure 8. *De novo* clustering of EHV-1 genes**

The clustering of EHV genes is based on the normalized canonical transcript counts (reads spanning from the canonical TSS tot the TES), compared to the traditional kinetic classification. The rows show the *de novo* cluster memberships, while the color represent the kinetic classes.

**Supplementary Figure 9. Dynamics of splice isoforms**

This figure presents the splicing dynamics within EHV-1 for the genes (a) ORF65, (b) ORF44. The right side of each panel shows the transcript annotations, along with their parent genes and genomic locations displayed below them, with light red indicating positive-strand genes and light blue indicating negative-strand genes. The analysis focused on transcripts that matched exactly, allowing a deviation of +/- 2 nucleotides (nt) for splice junctions and +/- 10 nt for the start and end positions of transcripts. Asterisks indicate the CAGE-Seq significance level for each reference transcript. On the left side of each plot, the temporal trends of these transcript isoforms are depicted, with averages and standard deviations (SD) calculated for each time point post-infection, based on read count data from the dcDNA-Seq. Each data point is linked by lines to demonstrate the progression over time. The transcript isoforms, are color-coded according to their distinct isoforms, with these colors matching those used for the points and lines in the left panel. The isoform counts were normalized against the total number of isoform counts for each gene in each sample to calculate the ratio of each isoform. Isoforms on the right side are colored grey, if they not originate from the given gene and thus were not included in the isoform ratio calculation.

**Supplementary Figure S10. Dynamics of total viral reads.**

This figure shows the total coverage of the viral genome, calculated from reads, whose orientation were determined using either the 5'-adapter, or the polyA-tail. The sub-plots show the data on different scales, limited to a read count of: (A) 5,000; (B) 500; (C) 50; and (D) using no predefined limit.

**Supplementary Figure S11. Comparison of replication origin-associated transcripts**

This illustration compares the sequences of raRNAs [(a) CTO-S; (b) NOIR] from EHV-1 with those of two PRV strains (Kaplan and MdBio).

## **Legends to Supplementary Tables**

**Supplementary Table S1. Read counts**

**Supplementary Table S2. Concentrations of total and poly(A)-selected RNAs**

**Supplementary Table S3. Identification of the EHV-1 immediate-early gene through the inhibition of protein synthesis**

In this experiment, we utilized two concentrations of CHX (20 and 100 mg/ml) and observed the effects at two different incubation durations (6 and 8 hours). Our findings unequivocally indicate that ORF64 is the sole immediate early gene of EHV-1. The numbers refer to the number of the detected reads. Several EHV-1 genes exhibit low-level expression, a phenomenon that can be attributed to transcriptional noise, which becomes more pronounced with reduced CHX concentrations and extended exposure times.

**Supplementary Table S4. Novel EHV-1 transcripts**

This table summarizes previously annotated and novel transcripts, along with their TSSs, validated through multiple sequencing approaches, including CAGE, direct cDNA, and direct RNA sequencing. Details of transcript features and splicing events are organized across three sheets:

*Sheet A: Previously Published Transcripts* Lists previously annotated transcripts and incorporates their TSSs validated in this study.

*Sheet B: Novel Transcripts* Provides details of transcripts newly identified in this study through TSS-TES pairing.

*Sheet C: Comparison of LoRTIA and NAGATA for Intron Annotation* Lists introns annotated from dRNA sequencing data, comparing results obtained using LoRTIA and NAGATA.

Columns include:

● *Gene*: name of the associated gene.

● *Transcript name*: identifier of the transcript.

● *Transcript category*: type of transcript (e.g., monocistronic, multicistronic, antisense, or non-coding).

● *Start and Stop sites (5′- and 3′-ends), and introns*: genomic coordinates of the transcript and intron positions (if spliced).

● *Intron donor and acceptor sites:* genomic coordinates of the intron donor and acceptor sites.

● *Sequencing validation*: indicates the presence or absence of the transcript’s TSS as identified by dRNA-Seq (via NAGATA), CAGE-Seq, and dcDNA-Seq.

● *Confirmed putative RNAs*: highlights previously annotated putative transcripts that met the 5% relative abundance threshold in CAGE data (compared to the canonical transcript), which was applied to novel putative mRNAs.

**Supplementary Table S5. TSS, TES and gene abundances in the time-resolved dcDNA-Seq datasets and *de novo* clustering results**

This table consists of four sheets presenting EHV-1 gene-level abundances at multiple infection time points (1 to 48 hpi) along with their *de novo* clustering results. Each row includes the gene or its canonical TSS or TES, its preassigned kinetic class (IE/E/L/unknown), and the *de novo* cluster assignment. The columns labeled EHV-1\_[Time]\_[Rep] indicate viral read count–normalized abundances for each replicate (Rep) at the given time (Time).

● *Sheet A*: *TSS-Only:* Contains reads covering the canonical TSS, regardless of TES overlap.

● *Sheet B*: *TES-Only*: Contains reads covering the canonical TES, regardless of TSS overlap.

● *Sheet C:* *Gene Dynamics (TSS+TES):* Contains only reads overlapping both the canonical TSS and TES.

● *Sheet D*: *Non-Normalized*: Contains all raw dcDNA-Seq counts, including TSS and TES. Empty fields represent cases where either the TSS or the TES did not overlap.