**Reviewer #1:**

***1)*** *While it is clear why the authors perform CAGE, it remains unclear what the advantage is of now performing dcRNA-seq rather than direct RNA sequencing, which the authors already previously performed. Furthermore, they already published an article performing a re-annotation of the EHV-1 transcriptome (Tombácz, Dóra et al. Heliyon, Volume 9, Issue 7, e17716) but no comparison to this re-annotation is performed. It is thus unclear how their new annotation (the second within a year) compares to the previous one. For instance, they find that “Specifically, 251 transcripts received the highest level of support (\*\*\*), indicating robust validation by CAGE-Seq. Medium support (\*\*) was found for 47 transcripts, while the lowest level of support (\*) was seen in 51 transcripts (Supplementary Table 2).” But it is unclear whether these are novel transcripts detected or previously annotated ones, in particular ones from their previous study.*

We thank the reviewer for the detailed comments and the opportunity to clarify the methodological choices and novel contributions of our study.

Direct RNA sequencing (dRNA-Seq) is valuable for identifying splice sites and transcription end sites (TESs) due to the use of oligodT primers and the low variance in TESs of alphaherpesviruses. However, this technique lacks accurate 5' end information due to 5' truncation caused by motor protein stalling, which limits its reliability in identifying transcription start sites (TSSs). Additionally, dRNA-Seq is prone to various types of errors compared to dcDNA-RNA-Seq (e.g., base-calling issues and read truncation), motivating us to apply a combined approach. Integrating both techniques allows us to achieve more reliable results by leveraging the strengths of each method.

In this study, we employed CAGE-Seq, which is widely accepted for its high resolution in TSS identification, specifically to accurately determine TSSs, complementing the TES and splice site information obtained from dRNA-Seq. While our previous publication focused on annotating canonical transcripts, the current study allowed us to identify additional transcript isoforms and novel TSSs. However, the primary novelty of our manuscript lies in investigating the temporal dynamics of viral transcription. Here, dcDNA-Seq proved particularly suitable, as it avoids amplification steps and generates full-length reads with true TSSs, thanks to the presence of adapters at the ends of reads (no adapters are used for dRNA-Seq). Thus, dcDNA-Seq provides a more accurate depiction of transcriptional timing.

We have indeed compared our current findings with previously published data, as shown in Supplementary Table 2. In the revised manuscript, we will address the points of unclarity noted by the reviewer, particularly regarding the distinction between novel and previously annotated transcripts. Balázs (Torma?)

***2)*** *Often the conclusions presented in the article are only substantiated with some references to figures or supplementary tables without any explanation on how they come to this conclusion. This applies e.g. to “Subsequently, we performed long-read dcDNA sequencing. Our findings reinforced that orf64 is the sole IE gene in EHV-1 (Supplementary Table 1).” While in this table ORF64 is the only one with high numbers of reads (which are not normalized to transcript length by-the-way), they find reads for other ORFs at lower levels. So, they need to be clearer regarding why they do not consider the other ORFs IE. Notably, it is never explained which time-points would be considered immediate-early, early or late, making it even more difficult to follow the authors’ logic. Another example is the kinetic profiling of EHV-1 Transcripts, which is poorly described. In particular, they perform two different types of normalization (one to viral and one to host reads), without analyzing whether these provide different results or why. It also remains unclear whether the grouping of ORFs is based on the IE, E and L annotation or the new clustering they performed and whether both are consistent. They also do not explain what viral read count-normalized TSS-TES dynamics are for which they perform the clustering. Ezt nem értem.*

In the revised manuscript, we have added explanations to better substantiate our conclusions.

Regarding ORF64, the CHX treatment identified this gene as the sole immediate-early (IE) gene. We concluded this based on two observations from the data in Supplementary Table 1: (1) only ORF64 transcripts are expressed at high levels, and (2) increasing CHX concentration further amplifies the difference in expression between ORF64 and other genes. The remaining genes are expressed at such low levels that they likely represent transcriptional noise, which occurs because CHX does not fully inhibit protein synthesis. This is a normal phenomenon.

We are uncertain about the reviewer's suggestion (in parentheses) to normalize to transcript length, as we do not believe this is a requirement. However, we note that such normalization would further increase the difference in favor of ORF64 with regard to expression levels.

In the revised manuscript, we have specified the time points associated with IE, early (E), and late (L) transcripts as requested by the reviewer.

We acknowledge that the two types of normalization (to viral and host cell reads) produce different results. This difference arises because viral reads increase by several orders of magnitude during infection, while host cell reads decrease at a much smaller rate due to degradation caused by viral infection.

Finally, the ORF classification was based on transcriptional dynamics, and we have clarified this comparison in the revised version.

***3)*** *Obvious and important analyses are missing, this includes e.g.*

***3a.*** *A detailed analysis of the link between TSS and TES sites. They find that there is sometimes a discrepancy between the kinetics of TSS sites and TES sites, and they attribute this to the presence of multicistronic ORFs and alternative TES sites for a gene. However, since they performed long read sequencing they can match TSS to TES sites and explicitly analyze whether this is the explanation or is something else is going on.*

We have indeed identified transcripts that include TSS, TES, and intron information, allowing for a detailed mapping of these elements. However, for certain TSS sites, we were unable to find precisely validated transcripts. This is likely due to the low abundance of these transcripts or because they represent very long polygenic transcripts, which are often underrepresented in long-read RNA sequencing.

***3b.*** *The dynamics of spliced transcripts expression focuses only on the presence of spliced vs. unspliced transcripts but does not take into account the presence of different spliced variants for the same gene. This needs to be analyzed, whether there is just a general splicing trend during infection or whether particular spliced transcripts.*

We have revised the manuscript as suggested. Balázs (Torma?)

***Some other issues:***

***1)*** *The manuscript is written in a very confusing manner. In particular, the outline of the study is only described at the beginning of the results rather than at the end of the introduction, which makes the methods section difficult to understand. There is also no explanation on why they do not use direct RNA sequencing as in their previous study but need dcDNA-seq here.*

We have thoroughly revised the manuscript to improve clarity and have restructured the introduction to better outline the study’s objectives and methodology.

Regarding the choice of dcDNA-seq over direct RNA sequencing (dRNA-Seq) in this study, we opted for dcDNA-seq as it provides certain advantages in accurately identifying transcription start sites (TSSs) and full-length transcript structures. Unlike dRNA-Seq, which often truncates the 5' end due to motor protein stalling, dcDNA-seq avoids this issue by producing full-length reads with high reliability. This method also eliminates the need for amplification, preserving the original transcript proportions, which is crucial for accurately assessing transcriptional dynamics. Therefore, dcDNA-seq allowed us to achieve more robust and comprehensive results for the goals of this study.

***2)*** *Upper- and lower case is used inconsistently for ORFs and genes.*

We have revised the manuscript and now consistently use capital letters (ORF).

***3)*** *The noir/NOIR gene/transcript appears to be a novel transcript from their previous study, but that is never explained.*

We have revised the manuscript to include this information. Balázs

***4)*** *The font size in figures is generally very small, making it often almost impossible to discern the ORFs they refer to in the manuscript and at least partially confirm their conclusions. Furthermore, x- and y-axis labels are often missing as well as color legends within the figures. While sometimes this is explained in the caption to the figures, it is not done so consistently and makes it difficult to understand the figures.*

We have revised the manuscript to improve its quality and readability. Balázs

***5)*** *Their “in-house developed R pipeline” should be made available either as supplement or Zenodo/Github.*

We have made it available …. Balázs

***6)*** *It is unclear why they explicitly note some R packages but not the “other R-packages from the Bioconductor repository”.*

A Balázs

***7)*** *I do not understand the point of Figure 6.*

Figure 6 illustrates the extensive transcriptional overlaps, the detection of which required long-read RNA sequencing. These overlaps are significant, as they likely indicate interactions between transcriptional machineries that may play a role in gene expression regulation. We have expanded on this topic in the revised manuscript.

**Reviewer #2:**

*This is a fine sequencing paper for equine herpesvirus. It is well written and is in line with the large volume of stellar sequencing manuscripts published by this group. I appreciate their work. However, it would be nice to use proteomic to define the alternatively spliced variants of the transcriptome of herpesviruses. So a transcriptome and proteome manuscript would be even better.*

We thank the reviewer for the valuable comments and constructive feedback on the manuscript. The suggestion to incorporate a proteomic analysis is well-noted, and we fully recognize the importance of such an approach. However, including this analysis would significantly broaden the scope of the current study. Therefore, while this manuscript does not intend to address proteomics, we plan to consider this in a dedicated future study, where the complexity of proteomic data can be thoroughly explored. Thank you once again for the helpful insights, which have contributed to refining the research direction.

So. We did include a set of new transcripts, as because the new method that we used here, CAGE-Seq, validated them. We found these transcripts in our previous article but as the result of strict filtering, we excluded them from the final list. Now, as some of these were supported by the CAGE-seq, we now include them. These had to share a TES with the previously described transcripts, as the was CAGE only used to find novel 5-prime sites. Only these reads were used that had a "correct" 5-prime adapter (as per LoRTIA) and their 5-prime ends were positioned in the CAGE TSS clusters identified with CAGEFighteR (and shared their 3-prime sites with the previous transcripts). Then these reads were further clustered by the 5-prime sites, as some CAGE clusters were too large (some of them exceeding 200 bps), using these dcDNA 5-prime end counts (and using mclust R package).

Then these reads were combined into transcripts, based on their TES, shared with (previous annot) and the thus created 5-prime cluster's peak position. Finally, these were filtered for at least 5 read counts in all the dcDNA timepoint samples.