FINAL REPORT

The major goal of the project was to determine the global transcriptomic profiles of a number of viruses belonging to different virus families including human & veterinarian pathogens. Specifically, we planned to characterize and assemble catalogues of the structural properties of viral RNA molecules, including the annotation of transcript isoforms [splice and transcription start site (TSS) and end site (TES) variants], the mono-, bi,- and polycistronic structure of the transcripts, and in some cases, the epigenetic modifications, and RNA editing. We also aimed to determine the time-course dynamics of the global transcriptomes in several viruses and the effect of the viral infection on the gene expression of the host cells.

We have published the results of this project in 25 articles (11 of them are in D1-ranked journals and 4 in Q1-ranked journals) of which in 20 I am first/last/corresponding author. In addition, we have an article accepted for publication in a Q1-ranked, and another article conditionally accepted in a D1 journal. Moreover, we have completed the laboratory work for several manuscripts. The cumulative impact factors of the publications (only *published* and *accepted*) supported by this grant is 217.515 according to the latest impact factor list.

The following viruses, cell lines and techniques were applied during the study:

I. Viruses
   A. DNA viruses
      - *Herpesviridae* family: Herpes simplex virus type 1 (HSV-1), Varicella Zoster virus (VZV), Pseudorabies virus (PRV), Equid alphaherpesvirus type 1 (EHV-1), Bovine alphaherpesvirus type 1 (BoHV-1), Human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), Kaposi sarcoma-associated herpesvirus (KSHV)
      - *Poxviridae* family: Vaccinia virus (VACV), Monkeypox virus (MPXV)
      - *Asfariviridae* family: African swine fever virus (ASFV)
      - *Baculoviridae* family: *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV)
   B. RNA viruses
      - *Coronaviridae* family: Severe acute respiratory syndrome-associated coronavirus (SARS-CoV-2)
      - *Orthomyxoviridae* family: H1N1 subtype of Influenza A virus (IVA)
      - *Flaviviridae* family: Zika virus (ZIKV), West Nile virus (WNV)
      - *Bunyaviridae* family: Crimean-Congo hemorrhagic fever virus (CCHFV)
      - *Picornaviridae* family: Coxsackievirus ‘group B serotype 5 (CVB5)
      - *Rhabdoviridae* family: Vesicular stomatitis Indiana virus (VSIV)

II. Cells, cell lines (for propagation of viruses)
   - Vero (HSV-1, SARS-CoV-2, VSIV, CVB5, ZIKV, WNV, CCHFV)
   - Pig kidney 15 - PK-15 (PRV)
   - Rat glioma - C6 (PRV)
   - PC12 (PRV)
   - Madin Darby bovine kidney - MDBK (BoHV-1)
   - Ovine kidney - OK (BoHV-1)
   - Rabbit kidney 13 - RK-13 (EHV-1)
   - Human lung fibroblast cells - MRC-5 (HCMV)
   - Akata (EBV)
   - KSHV-positive primary effusion lymphoma cells - iBCBL1-3xFLAG-RTA102 (KSHV)
   - CV-1 (VACV, MPXV)
   - clonal isolate of *Spodoptera frugiperda* - Sf9 (AcMNPV)
   - Madin-Darby canine kidney - MDCK (IVA)
   - Human glioblastoma - T98G (VSIV)
   - Porcine alveolar macrophage - PAM (ASFV)

III. Techniques (details can be found in the methods sections of our articles listed below)
   A. Sequencing

- Long-read sequencing (LRS)
  - Oxford Nanopore Technologies, MinION device: amplified cDNA-sequencing (acDNA-seq), direct cDNA-sequencing (dcDNA-seq) with or without Terminator handling, direct RNA-sequencing (dRNA-seq), Cap-selection (Cap-seq), Methyl-seq, RNA modification detection, LRS after terminator treatment
  - Pacific Biosciences, RSII and Sequel platforms: Isoform sequencing (Iso-seq), non-amplified sequencing (dcDNA-seq)
- Synthetic long-read sequencing (SLRS)
  - Illumina MiSeq instrument combined with Loop Genomics (now part of Element Biosciences) LoopSeq approach
- Short-read sequencing (SRS)
  - Illumina MiSeq instrument: acDNA-seq, Cap analysis of gene expression (CAGE-seq), Ribo-seq, ChIP-seq, Methyl-seq (bisulfite conversion), SRS after RNase R treatment

B. Additional laboratory techniques: quantitative real-time PCR (qRT-PCR), microscopy (fluorescent and confocal), molecular cloning (CRISPR/Cas9 technique)

C. Bioinformatics: SMRT Link, Blasr, Albacore, Guppy, GMAP, minimap2, STAR, IGV, Geneious, NanoPipe, LoRTIA, R packages and CIRI were also used, among others.

We have carried out LRS (in many cases combined with SRS) for the examination of the 'static' RNA profile of each above mentioned virus (altogether 12 DNA and 7 RNA viruses). In the grant proposal, we planned to examine transcriptomes of four additional viruses [Human herpesvirus 6 (HHV-6), Measles, Mumps and Rubella viruses], however we could not obtain sufficient read counts (yield) needed for a publication. Instead of these viruses, we have analyzed the KSHV (which is a γ-herpesvirus, similar to the HHV-6), the SARS-CoV-2 (a novel virus, which caused Covid-19 pandemic during the project), the MPXV (which caused an outbreak during the project) and the ASFV (because of the genome organization of this virus is similar to those of Vaccinia virus). We have carried out LRS using ONT MinION device and/or PacBio Sequel and RSII instruments and qRT-PCR for the kinetic analysis of the transcriptomes of HSV-1, PRV, BoHV-1, EHV-1, VACV, MPXV, AcMNPV, SARS-CoV-2, and VSIV. SLRS approach was applied for validation of the kinetic properties of BoHV-1 transcripts, while - beside LRS - we used the conventional SRS technique for ASFV, PRV and EHV-1 analysis. We have also examined the effect of BoHV, EHV-1, PRV, VACV, MPXV, AcMNPV, SARS-CoV-2 and VSIV infection on various host cells. QRT-PCR was also applied for validation. We have generated mutant viruses using homologous recombination (HRT) and CRISPR/Cas9 techniques. Using HRT, we were able to knock out 'key' viral genes encoding transcription regulators. We used the CRISPR/Cas9 method for modifying viral promoters (gene expression enhancement) with the aim to obtain evidence for the interaction between the transcription machineries. The effect of various gene mutations on the global expression profile of viruses were quantified using LRS [differential gene expression analysis (DE) between the expression levels of mutant and wild-type viruses]. The TSS positions of the novel RNAs obtained by our LRS approaches were validated using several auxiliary techniques including CAGE-seq, direct RNA sequencings, etc. We carried out integrated approaches using datasets produced by us and others in HSV-1, HCMV, and VZV. Ribo-seq and ChIP-seq data were also used for our VZV study. We have carried out RNase R treatment for the experimental examination of circ RNAs of viruses (HHV-6 and KSHV, because these RNAs can occur in intron-rich genomes). Our most important results are as follows:

TRANSCRIPTOME PROFILING OF VIRUSES We published our results on transcriptome profiling of eight large DNA viruses: HSV-1[1]; PRV[2]; BoHV-1[3]; HCMV[4]; EBV[5]; VACV[6]; ASFV[7]; AcMNPV[8]. We also published our results on RNA profiling of SARS-CoV-2[9] as well as the transcriptomic data of six other small RNA viruses[10] (VSIV, IVA, ZIKV, WNV, CCHFV, CVB5). A manuscript about our results using OMICS approaches for the analysis of EHV-1 is accepted for publication in Heliyon[11]. An analysis paper on the global transcriptome of MPXV has been resubmitted to Scientific Data after the requested minor revisions[12]. We have analyzed the epitranscriptome of AcMNPV using dRNA-seq data as well as bisulfite sequencing (5-mC methylation)[13]. Writing of manuscripts about

the results of integrated technical approach for VZV and KSHV analyses is in progress (data from these experiments are party used in our recent preprint article[14]). Our examinations on the transcriptomes of these viruses identified hundreds of ***RNAs***, including ***mRNAs, non-coding transcripts, multi-splice RNAs, mono-, bi-, and polycistronic RNAs, and complex transcripts***. The most important novel transcripts (e.g. CTO-S, NOIR-1, AZURE, ELIE, OriS-RNA, etc.) obtained by LRS were validated by other techniques (e.g. qRT-PCR, Northern-blot, sequencing other than LRS). We have also identified ***novel TSS and TES*** positions. We found that in many cases, longer 5′-UTRs (long TSS variants) harbor upstream ORFs[4,6,13], which have been shown (Kronstad et al., 2013) to represent an alternative mode of translational regulation. Our results shown that the transcripts of the examined large dsDNA viruses are ***much more complex*** than it was previously known. Our work demonstrated that the viral transcriptomes form complex networks of coding and non-coding RNAs which may interact with each other at their overlapping regions during RNA synthesis. In this project, we were able to detect a large number of ***novel transcriptional overlaps and transcriptional read-throughs*** between the proximal and distal genes. We have shown that each divergently-oriented gene pair produces overlapping transcripts. Our data provide evidence that the parallel overlaps are much more frequent then it was previously known. We found that all of the examined DNA viruses produces very long (>10kb) transcripts which may have role in the regulation of gene expression. We have also demonstrated that the presence of transcriptional overlaps and the variety of TSS and TES isoforms are universal within the examined virus families, however their frequencies vary between the viruses. For example we detected an extreme variability of TES variants in VACV[6], AcMNPV[8] and MPXV[12], which is a less frequent feature of herpesvirus transcripts. Beta - (HCMV) and gammaherpesviruses (EBV, KSHV) have much more spliced transcripts than the members of alphaherpesviridae family [HSV-1[1], PRV[2], VZV (**Fig. 1**), EHV-1[11], BoHV[3]], however, our detailed analyses shown that splicing and alternative splicing events are more common in these latter viruses than it was previously known. Splicing does not occur in VACV, MPXV, AcMNPV and the examined RNA viruses. Our data also demonstrate the existence of a ***pervasive antisense RNA expression throughout the entire genomes*** of DNA viruses. Analyses of RNA viruses[9,10,15] shows that their RNA complexity is less extensive, some of them (e.g. VSIV, SARS-CoV-2) have genes with variable TSSs. Our investigations revealed that, although the VSIV and the SARS-CoV-2 genomes are simple, their transcriptomic architectures are relatively complex. The VSIV RNA profile was analyzed in two different cell types and we shown that VSIV transcripts vary in structure and ***exhibit differential gene expression patterns in the two examined cell lines.***

A number of transcripts with proven or putative regulatory role, mapping close to or overlapping the replication origins (Oris) and the nearby transcription activator genes, have been described earlier in herpesviruses. ***Novel classes of replication-associated transcripts*** were also discovered by our investigations[16]. Beside our earlier publication[16], we have carried out multiplatform OMICS approach as well as data integration from other research groups to discover additional transcripts located around the Oris of herpesviruses[14]. We identified novel long non-coding RNAs (lncRNAs), as well as splice and length isoforms of mRNAs and lncRNAs. Furthermore, our analysis disclosed an intricate meshwork of transcriptional overlaps at the examined genomic regions. Our results suggest the existence of a '***super regulatory center*** 'which ***controls both the replication and the global transcription*** through multilevel interactions between the molecular components.

For the ***dynamic profiling*** of viral transcriptomes, various techniques (multiple library preparation approaches for LRS, SLRS and qRT-PCR) were applied. Using these approaches, we were able to ***categorize*** the HSV-1[17] and BoHV-1[18,19] ***transcripts into kinetic classes***. We also grouped the TES, TSS and splice isoforms and cistronic variants of HSV-1 transcripts. We applied cycloheximide treatment, which is a translation inhibitor, for the identification of the immediate–early (IE) genes[18,19]. With this approach, we have shown that the BoHV-1 ***circ gene*** is expressed in an IE kinetic. The VACV[20,21] and ASFV[22] RNAs were also analyzed according to their expression level of various time points after infection. The abundant VACV transcripts were kinetically categorized according to their expression dynamics into five distinct groups ('early-up', 'late-up', 'constant', 'late-up-down', and 'mid-up-down'). Based on the dynamic pattern of viral genes (ORFs), ***we created*** five ***distinct***

clusters (‘early-up-down’, ‘moderate-early-up’, ‘mid-up-down’, ‘late-up’, and ‘constant). We have also analyzed the dynamic expression pattern of EHV-1[11], SARS-CoV[9], MPXV[12] and VSIV[15] genes. We identified a large number of lncRNAs from which we performed kinetic analyses for the followings: CTO-S, NOIR-1 and AZURE and we obtained that CTO-S and NOIR-1 are expressed at a low level at the first four hours of post-infection while AZURE is expressed at high level at 4h of post-infection. According to these results, CTO-S and NOIR-1 are categorized as late genes whereas AZURE as an early non-coding gene.
Overall, our results demonstrated that the transcripts of DNA viruses form an extremely complex pattern of overlaps, and that the entire viral genomes are transcriptionally active. In most viral genes, if not in all, both DNA strands are expressed. Our results on virus transcriptome analysis using LRS approaches was also published as a review article in Trends in Microbiology[23].

ANALYSIS OF THE EFFECT OF VIRAL INFECTION ON HOST CELLS We have carried out detailed analyses on the effect of infections caused by various viruses on cell lines. We published our results on the effect of BoHV-1 infection on MDBK[24], the effect of VACV infection on CV-1[25], the VSIV on Vero and on T98G cells[15]. As a result of the BoHV-MDBK experiments we identified a total of 8342 host genes that produced sufficient read count for the analysis**.** Applying DE analysis with a 0.01 false discovery rate threshold, ***we identified*** 686 genes among the 8.342 ***host genes that exhibited significantly altered expression levels*** during the entire period of virus infection. We identified four clusters (groups of genes) that were constantly upregulated, a cluster where the gene expression levels were steadily downregulated throughout the entire course of virus infection, and finally, one group that showed initial upregulation followed by downregulation[24]. We have also examined the time-varying transcriptome profile of CV-1 cells during VACV infection, applying LRS datasets. In concordance with others recent proteomic results, our analysis revealed relatively few differentially expressed genes. We detected 768 highly expressed host genes and categorized them into five distinct clusters with respect to their responses to viral infection. These groups are as follows: ‘*early up genes’* where no or very low expression levels were observed before virus infection, but consistently high expression was detected at all later time points. Transcripts belong to the *‘early down’* category had a high expression level before virus infection and they not expressed or show low expression levels at later time points. The ‘e*arly up/down’* transcripts had no or low expression before virus infection, high expression from 1 h p.i. and no or low expression at later sampling points. The transcripts grouped to the *’mid up’* cluster had no expression before virus infection and peaked and plateaued at 2 or 3 h after infection. *Constant transcripts* had no significant changes in relative expression levels over the course of our experiments. The effect of SARS-CoV-2 infection on the host gene expression was also analyzed[9]. For this, we have developed novel bioinformatic pipelines. We were able to identify gene networks, which play roles in cellular and immune defense of the host organism. Our publication[12] on the OMICS analysis of MPXV – host cell interaction (dRNA-seq, dcDNA-seq and CAGE techniques) is accepted with minor changes and the revision was sent back to the journal. The effect of EHV-1 infection on RK-13 cells was also examined. **Figure 2** shows the six host gene clusters obtained by DE analysis on these datasets. The changes of the host gene expression during VSIV infection was also evaluated[15]. We found a significant difference in the effectiveness of viral infection between the Vero and T98G. Glia cells are much more sensitive to infection, while fibroblasts are more resistant. We detected 1.370 differentially expressed genes and 35 differentially expressed KEGG pathways between the cell lines. We detected 2.5 times more genes in the glial cells, whose expression trajectory was significantly affected by the viral infection (452 in glia, and 172 in Vero). From these, 137 were found to be significantly changed during rabies infection in mouse brain cells. This suggests that these genes are likely affected in other cell lines and host species, and thus should be the subjects of further investigation regarding the pathogenesis of vesicular disease in susceptible animals.

NOVEL METHODS We have developed several technical approaches and we were the first to integrate techniques that were commercialized during execution of the project. Among the novel developments, I emphasize the followings:

- **Novel software tool for the analysis of long-read transcriptomic datasets** Based on the obtained LRS datasets, ***our group has developed a pipeline***[26] for the analysis of LRS transcriptome data (name: ***LoRTIA*** - **Lo**ng-read RNA-Seq **T**ranscript **I**soform **A**nnotator toolkit).
- **Novel approach for single nucleus transcriptome analysis** We have developed a novel approach, which allows the examination of the transcripts within the nucleus from frozen tissues. This work has been carried out in a collaboration[27] with the Cornell University, New York City and other collaborators. We adapt this technique in viral transcriptome studies, using the Parse Bioscience Evercode Kit. We are working on the publication of the obtained data.

PROBLEMS AROSE DURING THE IMPLEMENTATION OF THE PROJECT The major problem during the implementation of the project were the pandemic lock-downs and winter/summer closures at the University. It is specially affected the most labor-intensive parts of our work, including molecular cloning, CRISPR/Cas9 technique, etc. The experimental part of these works has been completed. We have been analyzing the obtained results using bioinformatic approaches, we are currently working on the articles.

IN SUMMARY, during this project primarily due to the applied state-of-the art technical approaches, we identified a large number of novel viral transcript isoforms. We have discovered novel, potential protein coding, and non-coding RNAs, of which one of the most interesting transcripts overlap or initiated within OriS. These overlaps potentially indicate an interference between the replication and transcription machineries. We have discovered an enormously complex network of transcriptional overlaps in each of the examined viral families, which implies an interaction between the transcriptional apparatuses. Our studies revealed important aspects of the regulation of viral transcription and replication. These results can be useful for developing antiviral therapies.

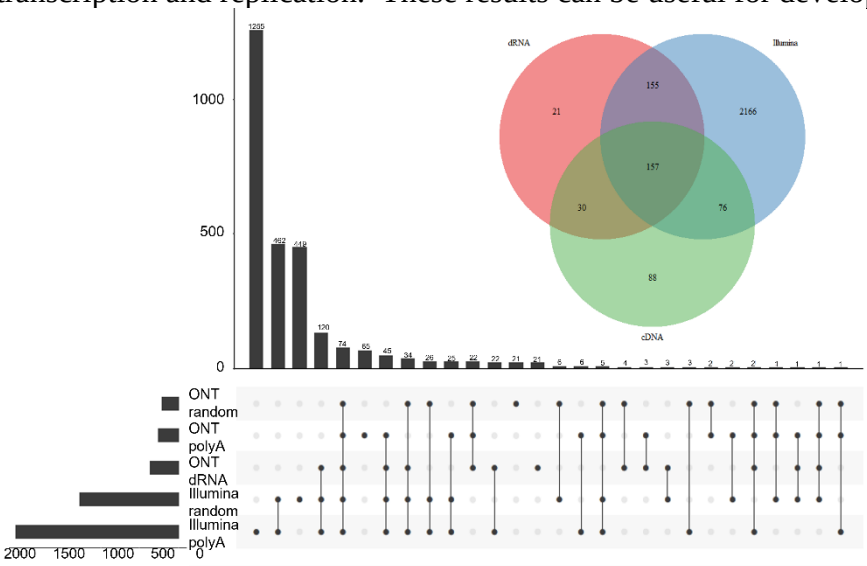

Figure 1*. Using an integrative technical approach, we detected many spliced VZV transcripts. The upset plot and the diagram show the techniques and the number of obtained splice isoforms). István Prazsák Ákos Harangozó, Balázs Kakuk, Gábor Torma, Zsolt Csabai, Gábor Gulyás, Dóra Tombácz, Zsolt Boldogkői: Multiplatform and integrative approach to assess the Varicella Zoster Virus transcriptome (Before submission)*

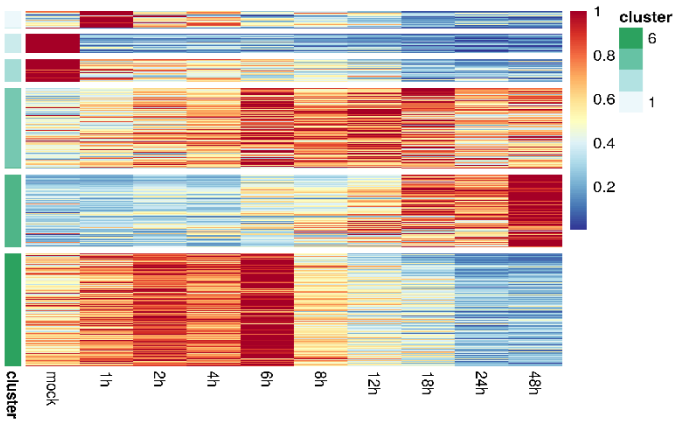

Figure 2. *Altered host gene expression caused by EHV-1 infection. The identified genes were clustered into six groups n the basis of their kinetic properties. Clusters 1-3: immediate-early response genes (transcription factors, components of antiviral signaling pathways), which are up- or downregulated at the very early stage of infection. Clusters 4-6: late response genes. Dóra Tombácz, Zoltán Maróti, Ákos Dörmő, Gábor Gulyás, Gábor Torma, Balázs Kakuk, Tibor Kalmár, Zsolt Csabai, Zsolt Boldogkői: Temporal transcriptome profiling of host cells infected by a veterinary alphaherpesvirus using multi-omics approach. (Before submission)*

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