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RNA-seq detects pharmacological inhibition of Epstein-Barr virus late transcription during spontaneous reactivation

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A B S T R A C T

The stepwise and sequential expression of viral genes underlies progression of the infectious life cycle. The Epstein-Barr virus (EBV) is both a tractable model for elucidating principles of transcription as well as a global health threat. We describe an experimental protocol and bioinformatics pipeline for functional identification of EBV true late genes, the last step of transcription prior to virion packaging and egress. All data have been uploaded to the Gene Expression Omnibus under accession code GSE96689. The key improvement over previous approaches is leveraging the sensitivity of RNA-seq to detect gene expression changes during spontaneous reactivation.

Specifications

Organism/cell line/tissue	GM12878 lymphoblastoid and MutuI Burkitt lymphoma cell lines
Sex	GM12878: female, MutuI: male
Sequencer or array type	Illumina HiSeq
Data format	Raw FASTQ and analyzed wig
Experimental factors	200 μM acyclovir vs. vehicle
Experimental features	Total RNA was purified for RNA-seq library construction
Consent	Not applicable
Sample source location	Not applicable

1. Direct link to deposited data

https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE96689

2. Introduction

Temporal control of Epstein-Barr virus (EBV) transcription drives transitions between different stages of the pathogenic life cycle [1]. In a manner similar to other herpesviruses, EBV lytic transcription proceeds via a sequential cascade: cellular signaling induces expression of immediate-early genes, immediate-early proteins transactivate early genes, early proteins promote DNA replication, DNA replication allows expression of late genes, and late gene proteins assemble virions for egress. No gold standard experiment, however, yet exists where the identities of EBV true late genes have been determined based on dependence for lytic DNA replication. Assignment as a late gene has generally been based on putative function or homology to other herpes proteins [1,2]. Experimentally, one intuitive approach would be to pharmacologically inhibit the viral lytic DNA polymerase and measure transcription changes genome-wide. Previous such attempts, however, have yielded confounding results. Microarray measurements showed that phosphonoacetic acid down-regulates all EBV genes, even early transcripts [2]. In contrast, PCR assays determined that acyclovir down-regulates no EBV genes at all [3]. The two conflicting experiments both initiated lytic transcription with IgG crosslinking prior to drug treatment [4]. In an attempt to improve upon previous results, we made two technical changes. First, we inhibit spontaneous reactivation naturally observed without external induction [5] in the hope that the effect of a pharmacological inhibitor would be more detectable. Second, we leverage the sensitivity of RNA deep sequencing (RNA-seq) to measure small transcriptional changes that are less accessible to other methods.

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Table 1
EBV genes regulated by acyclovir during spontaneous reactivation in GM12878 cells[a].

[a]Row shading indicates differentially regulated genes with a *p*-value < 0.05.
[b]Value not determined because of insufficient signal.

Table 2
EBV genes regulated by acyclovir during spontaneous reactivation in MutuI cells[a].

[a]Row shading indicates differentially regulated genes with a *p*-value < 0.05.
[b]Value not determined because of insufficient signal.

3. Experimental design, materials and methods

3.1. Cell culture

We maintained EBV-positive B cell lines at 37 °C with 5% CO_2 (v/v) in RPMI-1640 media containing 25 mM HEPES [5]. GM12878 [6] (Coriell Institute for Biomedical Research) and MutuI [7] cells were supplemented with 15% (v/v) and 10% (v/v) fetal bovine serum, respectively. For drug treatment, we added acyclovir (Sigma-Aldrich) to 200 μM from a 100 × stock solution in DMSO. Acyclovir- and vehicle-treated cells were cultured in parallel and treatment replenished with every addition of new media for at least one week.

3.2. RNA-seq

Total RNA was purified, libraries constructed, and sequences mapped as previously described [5]. We homogenized log phase cells with a QIAshredder (Qiagen), purified RNA with the RNeasy Mini Kit (Qiagen), converted RNA to cDNA with the Ovation RNA-Seq System V2 (NuGEN), sheared cDNA with an S2 Focused-ultrasonicator (Covaris), constructed libraries with either the Ovation Ultralow Library System V2 (NuGEN) or Encore NGS Library System I (NuGEN), and sequenced DNA with a HiSeq (Illumina). We mapped the first 50 bps of reads to an index containing both the human UCSC hg19 and EBV B95-8 [GenBank ID: NC_007605.1] genomes with Bowtie version 0.12.8 [8] and the parameter "-m 1" that limits analysis to unique sequences. Further data processing was performed with scripts written in the AWK programming language. The number of reads that map to each EBV bp were tallied and then normalized to every million reads that map to the entire index.

Read counts on the EBV genome were visualized with the Integrative Genomics Viewer [9,10]. The EBV B95-8/Raji genome sequence and annotations were loaded as fa and bed files, respectively [11], onto the browser. From our experimental counts of reads mapped to every EBV bp, we generated a 1 bp resolution wig file. The format consists of four columns: EBV chromosome identifier "chrEBV (B_95_8_Raji)", nucleotide position, nucleotide position + 1, and normalized mapped read count. This wig file was then visualized with the corresponding viral gene annotations.

We measured differential expression of viral transcription in GM12878 and MutuI cells upon acyclovir treatment by comparing independent and paired triplicate experiments. Nucleotide read counts for each lytic transcript were normalized to the total number of mapped reads and integrated only over exons spanning regions that did not overlap with another transcript based on version 6 of a published B95-8 annotation [11] (Tables 1 and 2). Regions corresponding to overlapping exons from more than one transcript were excluded from analysis. Genes were initially designated as either early or late based on known properties [2]. The differential expression significance threshold was set at a p-value < 0.05 as determined by a paired Student's t-test. Because of the small sample size within both the early and late gene sets, p-values were not adjusted for multiple testing. Expression level differences represent the mean fold change of biological replicates. Every data set yielded ~60–120 million mapped sequences with reproducible transcriptome profiles.

4. Data description

We demonstrate here that RNA-seq detects specific inhibition of EBV late genes by acyclovir during spontaneous reactivation. Transcriptome profiles show down-regulation of select EBV transcripts. In the GM12878 lymphoblastoid cell line, 14 out of 18 presumed late genes, but only 2 out of 17 presumed early genes, decrease expression upon acyclovir treatment (Table 1). Two early genes unexpectedly increase expression. Only 3 out of 18 presumed late genes decrease expression in the MutuI Burkitt lymphoma cell line (Table 2). MutuI samples displayed more variability within experimental groups compared to GM12878 samples, contributing to the lower number of statistically significant changes. In summary, enrichment for late genes among differentially expressed transcripts in two independent cell lines validates the specificity of this approach. We present our bioinformatic pipeline and experimental protocol for colleagues to investigate other inhibitors and cell lines in building a comprehensive classification of EBV true late genes.

Conflict of interest

The authors have no potential conflicts of interest to disclose.

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