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Epstein–Barr virus latency type and spontaneous reactivation predict lytic induction levels

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ABSTRACT

The human Epstein–Barr virus (EBV) evades the immune system by entering a transcriptionally latent phase in B cells. EBV in tumor cells expresses distinct patterns of genes referred to as latency types. Viruses in tumor cells also display varying levels of lytic transcription resulting from spontaneous reactivation out of latency. We measured this dynamic range of lytic transcription with RNA deep sequencing and observed no correlation with EBV latency types among genetically different viruses, but type I cell lines reveal more spontaneous reactivation than isogenic type III cultures. We further determined that latency type and spontaneous reactivation levels predict the relative amount of induced reactivation generated by cytotoxic chemotherapy drugs. Our work has potential implications for personalizing medicine against EBV-transformed malignancies. Identifying latency type or measuring spontaneous reactivation may provide predictive power in treatment contexts where viral production should be either avoided or coerced.

1. Introduction

The human Epstein–Barr virus (EBV) generates a global health burden. This lymphotrophic DNA herpesvirus evades the immune system by entering a latent phase in B cells that persists for life. Compared to lytic replication, during which about a hundred genes are expressed to amplify virus, latency restricts transcription to approximately a dozen or fewer genes [1]. EBV can immortalize B cells *in vitro* [2]. EBV is also associated with Burkitt lymphoma, Hodgkin lymphoma, nasopharyngeal carcinoma, gastric carcinoma, and other malignancies *in vivo* [1]. The requirement of specific latent genes for *in vitro* transformation strongly argues that viral infection contributes to oncogenesis *in vivo* [3]. Indeed, persistent infection correlates with ~1% of all cancer worldwide [4]. Detailed analysis of viral transcription during latency forms the foundational context for understanding how EBV interacts with cancer cells.

Latent forms of EBV express different sets of genes partially dependent on the developmental state of the cancer cell prior to immortalization [1]. While many exceptions to the rule exist, transcriptional programs generally stereotype to one of a few patterns. Type I latency limits expression to *EBNA1*, the gene encoding the DNA-binding protein responsible for latent replication and segregation, the *EBER* non-coding RNAs, and the *Bam*HI A rightward transcript (BART) RNAs. Type III latency further includes other *EBNA* isoforms and messages for the three *LMP* products. *BHRF1* is expressed under certain conditions [5,6].

Improving upon previous genomic technologies, RNA deep sequencing (RNA-seq) methods have illuminated novel details of viral transcription [7–12]. Increased sensitivity identified new transcripts and splice variants. Examination of Burkitt lymphoma cell lines uncovered both latent and lytic gene expression [7,8,11]. Lymphoblastoid cell lines (LCLs) display a wide range of spontaneous reactivation [9]. We asked if the dynamic range of lytic transcription in cell culture lines correlated with different EBV latency types. We further use measurement of spontaneous reactivation by RNA-seq to predict the induction response to cytotoxic chemotherapy drugs. Our work may have implications for personalizing medicine against EBV-transformed malignancies.

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2. Material and methods

2.1. Cell culture

We maintained Burkitt lymphoma cell lines at 37 °C with 5% CO_2 (v/v) in RPMI-1640 media containing 25 mM HEPES and 10% (v/v) fetal bovine serum. We maintained LCLs similarly except with 15% (v/v) fetal bovine serum. MutuI [13] cells grew under standard conditions [14]. Raji [15] (CCL-86) and Daudi [16] (CCL-213) cell lines were obtained from ATCC (Manassas, VA). The GM12878 [17] (GM12878) cell line was obtained from the Coriell Institute for Medical Research (Camden, NJ). Jeffery T. Sample (Pennsylvania State University) provided the KemI and KemIII [18] cell lines, Andrew I. Bell (University of Birmingham) provided the RaeI [19] cell line, and Bill Sugden (University of Wisconsin, Madison) provided the 721 LCL [20]. We generated MutuIII by prolonged passaging of MutuI in cell culture [13].

2.2. RNA-seq

We isolated RNA from 4×10^6 log phase cells homogenized with a QIAshredder spin column (Qiagen). Total RNA was purified by silica-based membrane affinity as packaged in the RNeasy Mini Kit (Qiagen). Preparations included the optional DNAse treatment step. Single primer isothermal linear amplification to cDNA [21] was achieved using the Ovation RNA-Seq System V2 (NuGEN) and 20 ng of RNA. 3 μg of cDNA was then sheared in a 40 μL volume using a Covaris S2 Focused-ultrasonicator. We prepared deep sequencing libraries by adaptor-mediated amplification [22] as packaged in either the Encore NGS Library System I (NuGEN) or Ovation Ultra-low Library System V2 (NuGEN).

Each library was sequenced on a HiSeq (Illumina). 50 bp reads were mapped using Bowtie [23] to an index containing both the human hg19 and EBV reference [GenBank ID: NC_007605.1] genomes. Parameters allowed for up to two mismatches and only considered reads that mapped to a unique sequence. The number of hits at each base was counted and then normalized per million mapped reads. RNA-seq profiling for every cell line was performed with two or three biological replicates and yielded ~30–100 million mapped sequences each experiment with reproducible transcriptome profiles.

2.3. Viral reactivation

We induced lytic replication of EBV with chemicals [14] and measured reactivation by staining for the immediate-early lytic transactivator BZLF1 using the paraformaldehyde-methanol method [24] with a BZ1 antibody (Santa Cruz Biotechnology) and goat anti-mouse IgG-FITC (Santa Cruz Biotechnology). Cells were treated for three days with 100 μM bendamustine (Sigma–Aldrich or Millipore), 1 μg/mL gemcitabine (Sigma–Aldrich), or 20 nM romidepsin (Selleck Chemicals).

3. Results

3.1. Dynamic range of spontaneous reactivation

We began by asking what a strictly latent EBV transcriptome looks like as measured by RNA-seq. To do so we examined the Raji [15] and Daudi [16] cell lines, which adopt latency type III and I, respectively. Both express very few viral gene products either spontaneously or in response to stimuli: these lines contain little early antigen under basal conditions, chemical treatment only weakly increases early antigen production, and chemical treatment cannot generate viral capsid antigen production [25,26]. By the above metrics, Raji cells are generally more latent than Daudi cells. Mechanistically, Raji genomes have a deletion in the *BALF2* gene necessary for progression through lytic replication [27]. Daudi genomes have deletions in other genes [28], although the source of the lytic replication defect is less understood. Both Raji and Daudi transcriptomes predominantly consist of signals from latent genes (Fig. 1). Identified Raji transcripts include *EBNA2*, *BHRF1*, and *LMP1*. Other overlapping *EBNA* exons are also expressed. In Daudi cells, the strongest signal is the *BHRF1* message. Our results resemble analysis performed by others with the same lines [11]. Signals from lytic gene expression generally peak at ~0–2 counts per million mapped reads. These profiles act as a baseline for defining a latent transcriptome.

Cell lines with the same latency type display a broad range of spontaneous reactivation. We measured the transcriptomes of MutuI, KemI, and RaeI cells as a sampling of type I latency lines (Fig. 2). In agreement with previously published data [7], we detected widespread lytic transcription in the MutuI line. *BHLF1* displays the strongest signal. Initiation of lytic DNA replication requires transcription of this gene [29] and its expression level correlates with spontaneous reactivation [9]. Other predominant signals correspond to lytic transcripts as well. The KemI expression profile is similar: *BHLF1* displays the strongest signal amidst many lytic transcripts. The RaeI line, however, contrasts significantly and does not reveal lytic transcription on the same scale. The only notable signals consist of *BHRF1* and the BARTs. Trace levels of lytic gene expression resemble amounts observed with Raji and Daudi cells. RaeI transcriptomes therefore also reflect the more latent end of the dynamic range of spontaneous reactivation.

Type III latency lines display variability of spontaneous reactivation similar to type I lines (Fig. 2). The GM12878 LCL reveals a pattern consisting of the strong *BHLF1* signal and other lytic genes. Significant levels of latent transcripts, such as *LMP1* and the 5′ end of the *EBNA* message from the C promoter, are also observed. The 721 LCL, KemIII, and MutuIII lines, however, predominantly express latent transcripts such as *EBNA* messages, *BHRF1*, and *LMP1*. We were reluctant to apply quantitative metrics previously used to compare lyticness, such as percentage of viral compared to human reads, or strength of the *BHLF1* signal, to rank the lines we studied. While those approaches work when comparing transcriptomes with similar profiles and the same sequence [9], different levels of latent gene expression between viruses with different sequences confound analysis. We find that visual inspection, qualitatively comparing signals from latent and lytic genes, proves informative. Because of the large variability in levels of spontaneous reactivation within each latency type, bulk comparison of type I and type III lines does not illuminate any general trend between latency type

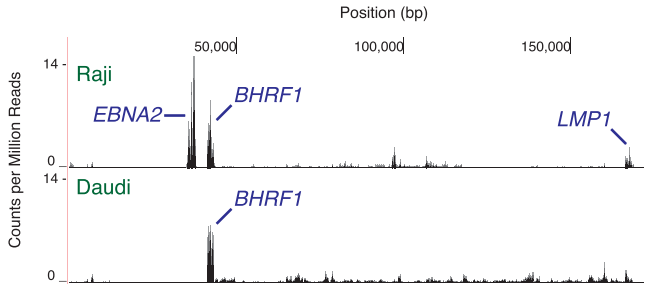

Fig. 1. Deep sequencing of EBV transcription in Raji and Daudi cell lines. The X axis denotes nucleotide position and the Y axis denotes the number of counts per million mapped reads. RNA signals with unambiguously assignable annotations are marked. *BHRF1*, *EBNA2*, and *LMP1* are latent transcripts labeled blue. One representative transcriptome from independent replicates is shown.