Mapping the Human Herpesvirus 6B Transcriptome

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ABSTRACT The “omics” revolution of recent years has simplified the study of RNA transcripts produced during viral infection and under specific defined conditions. In the quest to find new and differentially expressed transcripts during the course of human herpesvirus 6B (HHV-6B) infection, we made use of large-scale RNA sequencing to analyze the HHV-6B transcriptome during productive infection of human Molt-3 T cells. Analyses were performed at different time points following infection, and specific inhibitors were used to classify the kinetic class of each open reading frame (ORF) reported in the annotated genome of the HHV-6B Z29 strain. The initial search focused on HHV-6B-specific reads matching new HHV-6B transcripts. Differential expression of new HHV-6B transcripts was observed in all samples analyzed. The presence of many of these new HHV-6B transcripts was confirmed by reverse transcriptase PCR and Sanger sequencing. Many of these transcripts represented new splice variants of previously reported open reading frames (ORFs), including some transcripts that have yet to be defined. Overall, our work demonstrates the diversity and the complexity of the HHV-6B transcriptome.

IMPORTANCE RNA sequencing (RNA-seq) is an important tool for studying RNA transcripts, particularly during active viral infection. We made use of RNA-seq to study human herpesvirus 6B (HHV-6B) infection. Using six different time points, we were able to identify the presence of differentially spliced genes at 6, 9, 12, 24, 48, and 72 h postinfection. Determination of the RNA profiles in the presence of cycloheximide (CHX) or phosphonoacetic acid (PAA) also permitted identification of the kinetic class of each ORF described in the annotated GenBank file. We also identified new spliced transcripts for certain genes and evaluated their relative expression over time. These data and next-generation sequencing (NGS) of the viral DNA have led us to propose a new version of the HHV-6B Z29 GenBank annotated file, without changing ORF names, to facilitate trace-back and correlate our work with previous studies on HHV-6B.

KEYWORDS HHV-6B, RNA-seq, herpesviruses, transcriptomic

Human herpesvirus 6B (HHV-6) is a member of the genus Roseolovirus, within the Betaherpesvirinae subfamily of the Herpesviridae family. HHV-6B is acquired by greater than 90% of the population by the age of three and is the etiologic agent of Roseola, also called sixth disease or exanthema subitum (1). Roseola is usually resolved without any treatment and/or consequences, but febrile seizures have been observed in more than 10% of acute infections (2). HHV-6B reactivation is also frequent in hematopoietic stem cell transplant recipients, with serious medical consequences, including encephalitis and acute graft versus host disease (3). As with all herpesviruses, HHV-6B establishes latency following primary infection. During latency, most herpesviruses maintain their genome as episomes. The presence of HHV-6B viral episomes during latency awaits experimental confirmation. However, integration of HHV-6A/B into human genome replication and regulation of viral gene expression.
Chromosomes can be found in approximately 1% of the world population (4–6). HHV-6A/B can integrate in the telomeres of several distinct chromosomes (5, 7, 8). Chromosomally integrated HHV-6A/B can be inherited, resulting in individuals having at least one copy of the viral genome in every cell of their body. Viral integration into human telomeres has been suggested as an alternative form of latency. The repercussions of chromosomally integrated HHV-6A/B on an individual’s health are unknown, but we have recently demonstrated that these subjects are more likely to develop angina pectoris (9). Recent studies have also shown that chromosomally integrated HHV-6A/B may spontaneously express certain viral genes (U90 and U100), and these individuals have greater antibody responses against these viral genes than matched controls (10).

The HHV-6A/B genome comprises a 160-kb linear doubled-stranded DNA molecule (11, 12). A unique segment (U) is flanked by direct repeats (DR) of approximately 8 kb. The nucleotide sequences and annotations of HHV-6A/B genomes were obtained in the 1990s. The nucleotide sequence of the HHV-6A U1102 strain was the first published in 1995 (12). In this Ugandan strain, a total of 119 open reading frames (ORFs) were described, corresponding to 102 distinct genes. The nucleotide sequence was obtained following the sequencing of a library of viral genome segments cloned in plasmids, cosmids, and bacteriophages. Four years later, the nucleotide sequences of HHV-6B Z29 and HHV-6B HST, from Zaire and Japan, respectively, were published (11, 13). The nucleotide sequence of the HHV-6B Z29 strain predicts 119 unique ORFs comprising 97 unique genes, whereas the nucleotide sequence of the HHV-6B HST strain indicates 115 potential ORFs. These 2 sequences were determined by plasmid clones, overlapping PCR fragments, and sequencing. In 2013, Gravel et al. published the nucleotide sequence of a low-passage-number strain, HHV-6A GS (14). This strain was the first isolated HHV-6A in 1986 and originated from the United States. The nucleotide sequence was determined by Illumina sequencing, and gaps were filled by PCR. This sequence carries 88 putative genes. All annotated sequences were based on ORF analysis using conventional translation start and stop codons.

Innovative “omics” approaches in recent years have made high-throughput sequencing more available and less expensive, making large genome sequencing and RNA sequencing (RNA-seq) possible. In the last 2 years, a significant amount of data has become available, with the determination of numerous genomes, transcriptomes, translatomes, and proteomes for many species and pathogens. Herpesviruses have proven no exception, with many omics data sets available for cytomegalovirus, gammaherpesviruses, herpes simplex virus (HSV), and even HHV-6A/B. In recent months, a group from Israel reported novel and conserved genomic features for HHV-6A/B using RNA-seq and ribosome profiling (Ribo-seq) (15). Using these two techniques, they were able to accurately determine the translation initiation sites of previously annotated genes and to identify hundreds of new ORFs. Novel splice junctions were mapped, and novel highly abundant viral long noncoding RNAs were identified. They also proposed systematic annotations of the two viruses following their previous annotation of the human cytomegalovirus (hCMV) (16).

The objective of this study was to determine the RNA profile during the course of an active HHV-6B Z29 infection in the Molt-3 T-cell line. We also wanted to determine the RNA profile of HHV-6B Z29 infection in the presence of cycloheximide (CHX) and phosphonoacetic acid (PPA), two inhibitors that permit the identification of the kinetic class of each gene. Finally, we defined new spliced variants of several genes, identified new transcripts, and proposed a new annotation for HHV-6B Z29 that is built on the current and widely used reference sequence.

RESULTS

Coverage and splicing patterns. To explore the transcriptome of Molt-3 cells infected with HHV-6B, we performed an infection time course study with samples taken at 6, 9, 12, 24, 48, and 72 h postinfection. RNA was extracted and analyzed by RNA-seq. The 72-h postinfection sample was initially used to analyze the read coverage...
obtained using our RNA-seq protocol. As observed in Fig. 1, a coverage of 100× was obtained for most of the genome, indicating efficient transcription throughout the genome. Some regions showed very high coverage, e.g., the DR6 region, the forward direction of the U41-U42 region, and the reverse orientation of the U77 gene. In contrast, genomic repeat regions, exemplified by telomeric repeats, R2 and R3, showed less than 1× or no coverage, indicating that these regions are not efficiently transcribed.

**Relative expression and transcript kinetic class over time.** Using data from across the time course of infection, we generated a heat map showing the relative expression of each ORF described in the annotated HHV-6B genome (NCBI accession number AF157706). As shown in Fig. 2, we found that particular genes, such as U90 (IE1), were expressed in abundance throughout the course of infection. For other genes, such as U27, expression began at 9 h postinfection. Genes such as DR6 were only expressed from 48 h postinfection. To complete our analysis, we performed RNA-seq on RNA extracted from HHV-6B-infected Molt-3 cells in the presence of CHX, an inhibitor of protein synthesis (9C in Fig. 2). As shown in Fig. 2, the U90 (IE1) gene was highly expressed in the presence of CHX and can be classified as belonging to the immediate-early (IE) kinetic class. The DR3, B2, U2, U37, U38, U39, U40, U45, U59, U62, U64, B7, U86 (IE2), U94, and U95 genes were also expressed in the presence of CHX, categorizing these genes in the IE kinetic class. A similar experiment was performed in the presence of phosphonoacetic acid (PAA), an inhibitor of viral DNA polymerase (72P in Fig. 2). We characterized early (E) genes as those expressed in the presence of PAA but not in the presence of CHX. The U20, U22, U54, U55, U63, U74, and B6 genes were the most highly expressed genes in this kinetic class. Genes that were not expressed in the presence of CHX or PAA were categorized as belonging to the late (L) kinetic class. Examples include the DR1, DR6, U11, U18, and U100 genes.

The efficiency of PAA treatment was determined by quantifying viral DNA copies at 72 h postinfection in the absence or in the presence of PAA. As shown in Fig. 3A, a 95% reduction in viral DNA copy number per cell was observed in infected cells treated...
with PAA relative to infected and untreated cultures. Protein expression of one gene per kinetic class was evaluated in the presence of PAA. An immunofluorescence assay was used to detect the IE1 (U90), p41 (U27), and DR6 proteins. As shown in Fig. 3B, we detected the IE1 (U90) and p41 (U27) proteins in HHV-6B-Molt-3-infected cells 72 h postinfection in both the presence and absence of PAA. However, the DR6 protein was only detectable in the absence of PAA, confirming that DR6 is a late gene.

**Sequence analysis and comparison with the published sequence.** Next, we analyzed NGS data obtained from HHV-6B Z29 viral DNA and RNA-seq data obtained from HHV-6B-Molt-3 cells infected for 72 h. The sequence was first analyzed to identify nucleotide mismatches from the nucleotide sequence under NCBI accession number AF157706; 95 mismatches in the nucleotide sequence were identified (Table 1). Thirty-three mismatches (highlighted in orange) were previously found in the MF994829.1
sequence and reported by Finkel et al., while 12 additional mismatches (highlighted in pink) were restricted to the MF994829.1 sequence (17) (Table 1). Of these 95 mismatches, 26 were located in noncoding regions of genes annotated in the reference sequence, leaving 69 mismatches located in the coding regions affecting 74 amino acids of coding sequence (Table 1). Twenty of these 74 differences did not affect the amino acid coding sequence of the corresponding ORF (yellow), 16 differences were conservative mutations (green), 37 differences were nonconservative mutations, and one amino acid difference changed a tryptophan into a stop codon (blue) (Table 1). Six insertions were found in noncoding sequences, while two were found in the B9 coding sequence (Table 2). These insertions are present in the vast majority of HHV-6B sequences found in GenBank. They were previously identified in MF994829.1 (17), and five were referenced by Finkel et al. (15). One deletion was identified in the intergenic region between B6 and B7 (Table 2). This deletion removed a T from the original sequence at bp 119973. Comparison of all HHV-6B sequences found in GenBank supports that this is a genuine deletion. A number of reads cover this region, and most of the sequences found in GenBank have T deleted at this location. This deletion was also found in the MF994829.1 sequence and mentioned in the study by Finkel et al. (15, 17).

Our attention then turned to splice variants of the virus transcriptome. We used our RNA-seq data to generate a splice map in relation to the annotated genome (Fig. 4). A number of splicing events occurred around the U44 gene: long-range splicing events in the reverse orientation of the U77 gene, in the same orientation as the U95 gene, and the previously described multispliced transcripts of U86, U90, and U100 (Fig. 4). We first concentrated on the U7-U8 genes. U7 and U8 are described as two genes in the Z29 strain of HHV-6B (NCBI accession number AF157706) (Fig. 5A). Our RNA-seq data revealed that U8 is part of exon 1 of the U7 gene, since reads were found that spanned the exon-intron-exon region and consensus splicing acceptor and donor sites. The gene arrangement described here is similar to that of the HHV-6A U1102 U7 gene (NCBI accession number X83413) (Fig. 5A). To confirm these data, we designed PCR primers spanning the new intronic region and performed reverse transcriptase PCR (RT-PCR) on a new RNA sample from HHV-6B-Molt-3-infected cells (Fig. 5B). As shown in Fig. 5B, we observed a 350-bp PCR product corresponding to the unspliced version of the cDNA and a PCR product of 245 bp corresponding to the spliced version of the U7 gene. The two PCR products were isolated from the agarose gel, purified, and
## Table 1: Nucleotide mismatches detected in the HHV-6B sequence

| Genebank accession no. in HHV-6B | Mismatch | Protein amino acid difference | Location | Comment |
|----------------------------------|----------|-------------------------------|----------|---------|
| G152                             | T < C    | -                            | 331      | Non-coding |
| G154                             | A < C    | -                            | 331      | Non-coding |
| G156                             | A < C    | -                            | 331      | Non-coding |
| G157                             | G < A    | -                            | 636      | Non-coding |

Note: Some amino acids are conserved substitutions, while others are stop codons, and mismatches are located in ORF regions.
sequenced to confirm our findings. The DNA sequence is presented in Fig. 5C, showing the intronic region in lowercase. This new arrangement for U7 and U8 ORFs was described by Finkel et al. (15).

The U12-U13 region was the next region to be investigated. In the NCBI entry with accession number AF157706, the U12 gene is described as two exons separated by an intron (Fig. 6A). In the HHV-6A U1102 genome annotation, the U12 gene is also described as two exons separated by an intron, but the second exon is longer than that described for HHV-6B (Fig. 6A). HHV-6B Z29 is exceptional in this instance, as most other HHV-6B strains sequenced to date do not have this stop codon and resemble the HHV-6A version of U12 (18). The 72-h postinfection RNA-seq data revealed a limited
number of reads that covered the intron present between the two exons described for U12 (Fig. 6A). We confirmed these data using RT-PCR and sequencing, but the 104-bp PCR product was very faint, indicating very low abundance of this transcript (Fig. 6B and C). RNA-seq data also indicated that the first exon of U12 is part of the U13 gene (Fig. 6E). Splicing between exons 2 and 3 of U13 (Fig. 6D and E) and the splicing event in the noncoding region of U12-U13 (Fig. 6F and G) were confirmed by RT-PCR and sequencing. Therefore, U12 and U13 share both the noncoding exon 1 and exon 2 but have distinct third exons. This observation was partially described by Finkel et al. (15).

The RNA-seq data obtained for the region spanning U44 to U46 were of particular interest, since many reads and splicing events were observed. The HHV-6B Z29 annotation described the transcription of the U44 and U46 genes in the 5’ to 3’ direction, whereas the U45 gene was transcribed in the reverse direction (Fig. 7A). No intron was described for these three genes in either HHV-6B or HHV-6A (Fig. 7A). The presence of this new long intron in the U44 gene was confirmed by RT-PCR and sequencing of the PCR products (Fig. 7B and C). This new spliced U67 gene was not described in the annotated HHV-6A U1102 genome.

Other spliced transcripts. The region spanning U69 to B7 was investigated next. Our RNA-seq data revealed an intron that spanned more than 13,000 bp (Fig. 9A). This intron is part of a transcript that originated between the B6 and B7 genes and ended in the reverse orientation of the U69 gene. The presence of this intron was confirmed by RT-PCR and sequencing of the PCR products (Fig. 9B and C). Short exons flanked this intron, giving an ORF of 87 bp. While this transcript is of limited abundance, it was
FIG 6 U12-U13 region of HHV-6B. (A) Read analysis revealed a new arrangement for the U12 and U13 genes of HHV-6B. The AF157706-Z29 drawing represents the description found in annotated genome AF157706 for HHV-6B Z29. (B) PCR primers were designed to surround the splice region between the noncoding and coding exons (black arrows in panel A). PCR was performed as described in Materials and Methods. PCR products were analyzed on a 2% agarose gel. (C) PCR products were extracted from the gel, purified, and sequenced. Primer sequences are underlined, and the intron sequence is presented in lowercase. The intron sequence was cut (...) to fit the figure. (D) PCR primers were designed to surround the splice region between the coding exons of the U13 gene (black arrows). PCR was performed as described in Materials and Methods. PCR products were analyzed on a 2% agarose gel. (E) PCR products were extracted from the gel, purified, and sequenced. Primer sequences are underlined, and the intron sequence is presented in lowercase. The intron sequence was cut (...) to fit the figure. (F) PCR primers were designed to surround the splice region between U12-U13 exon 1 and exon 2 (black arrows). PCR was performed as described in Materials and Methods. PCR products were analyzed on a 2% agarose gel. (G) PCR products were extracted from the gel, purified, and sequenced. Primer sequences are underlined, and the intron sequence is presented in lowercase. The intron sequence was cut (...) to fit the figure.
present in the 48- and 72-h postinfection samples. It is currently unknown whether this spliced transcript translates into a functional protein or whether it is one of the non-coding RNAs (sncRNAs or lncRNAs) found in many herpesvirus genomes. In 2020, Finkel et al. reported the presence of at least 3 lncRNA in the HHV-6B genome (15). We confirmed the presence of lncRNA3 in our data and that this transcript was expressed as early as 9 h postinfection. This transcript was continuously expressed from 9 h up to 72 h postinfection and was expressed in the presence of PAA, suggesting that it was an early transcript whose expression did not require viral DNA replication.

DISCUSSION

The omics revolution of recent years has brought tremendous possibilities to the study of large genomes of any origin at different levels. Here, we determined the RNA profile of an HHV-6B Z29 active infection at 6, 9, 12, 24, 48, and 72 h postinfection and at 9 h and 72 h postinfection in the presence of CHX and PAA, respectively.

Using data from the 72-h postinfection RNA-seq, we constructed a splice map for the HHV-6B genome (Fig. 4). A number of splicing events were observed in the 68,000-
to 78,000-bp region of the genome, and all spliced events were oriented in the forward direction (Fig. 4). This observation coincided with the very high coverage obtained in the forward direction of the 69,000-bp region of the genome (Fig. 1) and could be explained by the fact that this location is the origin of replication (OriLyt) of HHV-6B. It was previously demonstrated that sequences deriving from around the OriLyt of rat and mouse cytomegalovirus, and herpesvirus simplex 1 (HSV-1) and HSV-2, can act as RNA primers for replication of the viral genome (19–21). Published studies report that miRNAs are in the OriLyt region. In 2011, Tuddenham et al. identified miRNAs and other small noncoding RNAs from HHV-6B in this region (22). In 2014, a group from Israel concluded that the finding of processed pri-miRNA in supraspliceosomes brought further support to the cross talk between the splicing and miRNA (19–21) processing machinery (23). Pri-miRNAs are the precursors of pre-miRNAs, which are themselves the precursors of miRNAs. Pri-miRNAs are hairpin structures of about 70 nucleotides, with a 5′-cap and a 3′-poly(A) tail at the respective extremities. Therefore, the large number of sequencing reads and the numerous splicing events that we observed in the vicinity of the OriLyt region likely represent pri-miRNAs, which can be processed to produce many miRNAs. A long noncoding RNA (IncRNA1) found in this region was previously identified as the most highly expressed RNA in HHV-6B (15).

From the RNA-seq analysis at each of the infection time points, we obtained a heat map of the relative expression of each ORF described in the annotated HHV-6B genome (accession number AF157706) (Fig. 2). From the 9-h time point in the presence of CHX and the 72-h time point in the presence of PAA, we could match the majority of ORFs described in the annotated genome to their previously defined kinetic class, immediate-early (IE), early (E), and late (L) genes (Fig. 2 and Table 3). We then compared the kinetic classes obtained with those from two previous studies conducted on HHV-6B (Table 3) (24, 25). Of the 97 ORFs described in the annotated genome, 50 ORFs were newly classified or classified as previously mentioned by other studies (Table 3).
Of the 47 remaining ORFs, 22 were classified as E genes, although these ORFs were previously classified as L genes in other studies (Table 3, green ORF). In general, the expression of E genes is not dependent on viral DNA replication (26). However, the accumulation of some E genes is enhanced by viral DNA replication; these genes are called early late genes. On the other hand, the expression of leaky late genes is delayed compared with that of E genes, and only true late genes can be identified by their “nonexpression” in the presence of PAA. The high sensitivity of RNA-seq used to analyze our data makes it difficult to distinguish early late genes from leaky late genes. Thus, our preference was to classify ORF transcripts that were detected 72 h postinfection in the presence of PAA as E genes. Nine ORFs from the remaining 25 were assigned to the L kinetic class, while other studies have classified these same genes as E genes (Table 3, orange ORF). Our results suggest these genes should be classified as L genes, as no transcription was observed for these genes at 72 h postinfection in the presence of PAA. Moreover, it was demonstrated that the hCMV homologs of HHV-6B U71 and U82 belong to the L gene kinetic class (Table 3) (27, 28). Four ORFs of the remaining 16 correspond to the B1, B5, B6, and B9 genes found only in the HHV-6B genome (Table 3, dark blue ORF). Conflicting reports exist regarding the kinetic class of these B genes. We have classified 3 genes as L genes, as they were not detected in the presence of PAA at 72 h postinfection (B1, B5, and B9). The fourth gene, B6, was classified as an E gene, since this transcript was found in the presence of PAA but was not detected in the presence of CHX. Previous studies classified B6 as an IE or a biphasic (IE and L) gene; however, these studies were conducted in different cell lines or with different HHV-6B strains (24, 25). Four genes out of the remaining 12 were assigned to the IE kinetic class, since they were expressed at high levels in the presence of CHX at 6 h postinfection (Table 3, gray ORF). These genes were previously assigned to the E or L kinetic class (24, 25). Two of the 8 remaining genes were previously classified as IE genes in other studies (Table 3, pink ORF). However, our study did not reveal any reads covering these genes in the presence of CHX (Fig. 2). Therefore, these genes were classified as members of the E kinetic class of genes. The remaining 6 genes were assigned

![FIG 9 ncRNA4 of HHV-6B](image-url)
| Order and Nomenclature | Type et al. (HCMV) | This study | HCMV | Gene | Kinetic class |
|------------------------|-------------------|------------|------|------|---------------|
| CN1                   | IME               | L          | IME  | -    | +             |
| CN15                  | IME               | L          | IME  | -    | +             |
| CN3                   | IME               | L          | IME  | -    | +             |
| CN16                  | IME               | L          | IME  | -    | +             |
| CN21                  | IME               | L          | IME  | -    | +             |
| CN22                  | IME               | L          | IME  | -    | +             |
| CN23                  | IME               | L          | IME  | -    | +             |
| CN24                  | IME               | L          | IME  | -    | +             |
| CN25                  | IME               | L          | IME  | -    | +             |
| CN26                  | IME               | L          | IME  | -    | +             |
| CN27                  | IME               | L          | IME  | -    | +             |
| CN28                  | IME               | L          | IME  | -    | +             |
| CN29                  | IME               | L          | IME  | -    | +             |
| CN30                  | IME               | L          | IME  | -    | +             |
| CN31                  | IME               | L          | IME  | -    | +             |
| CN32                  | IME               | L          | IME  | -    | +             |
| CN33                  | IME               | L          | IME  | -    | +             |
| CN34                  | IME               | L          | IME  | -    | +             |
| CN35                  | IME               | L          | IME  | -    | +             |
| CN36                  | IME               | L          | IME  | -    | +             |
| CN37                  | IME               | L          | IME  | -    | +             |
| CN38                  | IME               | L          | IME  | -    | +             |
| CN39                  | IME               | L          | IME  | -    | +             |
| CN40                  | IME               | L          | IME  | -    | +             |
| CN41                  | IME               | L          | IME  | -    | +             |
| CN42                  | IME               | L          | IME  | -    | +             |
| CN43                  | IME               | L          | IME  | -    | +             |
| CN44                  | IME               | L          | IME  | -    | +             |
| CN45                  | IME               | L          | IME  | -    | +             |
| CN46                  | IME               | L          | IME  | -    | +             |
| CN47                  | IME               | L          | IME  | -    | +             |
| CN48                  | IME               | L          | IME  | -    | +             |
| CN49                  | IME               | L          | IME  | -    | +             |
| CN50                  | IME               | L          | IME  | -    | +             |
| CN51                  | IME               | L          | IME  | -    | +             |
| CN52                  | IME               | L          | IME  | -    | +             |
| CN53                  | IME               | L          | IME  | -    | +             |
| CN54                  | IME               | L          | IME  | -    | +             |
| CN55                  | IME               | L          | IME  | -    | +             |
| CN56                  | IME               | L          | IME  | -    | +             |
| CN57                  | IME               | L          | IME  | -    | +             |
| CN58                  | IME               | L          | IME  | -    | +             |
| CN59                  | IME               | L          | IME  | -    | +             |
| CN60                  | IME               | L          | IME  | -    | +             |
| CN61                  | IME               | L          | IME  | -    | +             |
| CN62                  | IME               | L          | IME  | -    | +             |
| CN63                  | IME               | L          | IME  | -    | +             |
| CN64                  | IME               | L          | IME  | -    | +             |
| CN65                  | IME               | L          | IME  | -    | +             |
| CN66                  | IME               | L          | IME  | -    | +             |
| CN67                  | IME               | L          | IME  | -    | +             |
| CN68                  | IME               | L          | IME  | -    | +             |
| CN69                  | IME               | L          | IME  | -    | +             |
| CN70                  | IME               | L          | IME  | -    | +             |
| CN71                  | IME               | L          | IME  | -    | +             |
| CN72                  | IME               | L          | IME  | -    | +             |
| CN73                  | IME               | L          | IME  | -    | +             |
| CN74                  | IME               | L          | IME  | -    | +             |
| CN75                  | IME               | L          | IME  | -    | +             |
| CN76                  | IME               | L          | IME  | -    | +             |
| CN77                  | IME               | L          | IME  | -    | +             |
| CN78                  | IME               | L          | IME  | -    | +             |
| CN79                  | IME               | L          | IME  | -    | +             |
| CN80                  | IME               | L          | IME  | -    | +             |

The table compares the assigned kinetic classes obtained in this study with other studies and HCMV. The table includes columns for the order and nomenclature, type and study, the assigned kinetic class for this study, and the corresponding gene. Each row represents a different gene, and the kinetic class is indicated by a plus sign (+) in the appropriate column.
to the IE kinetic class because some reads were detected in the presence of CHX and PAA (Fig. 2) (Table 3, blue ORF). These observations suggest that these genes can be classified as both IE and E genes. However, it is also possible that RNA from these genes is packaged within the virion and does not represent nascent transcription (Table 3, blue ORF). This is of particular relevance to the U2 gene, which showed a marked abundance of transcripts at 6 h postinfection (Fig. 2). A comparison of gene kinetic classes between hCMV and HHV-6B gene homologs is also presented in Table 3. We observed that most HHV-6B gene kinetic classes determined in this study are the same as those of their hCMV homologs.

Next-generation sequencing combined with RNA-seq data bring us to deposit an updated version of the HHV-6B Z29 annotated genome. Our HHV-6B Z29 sequence was deposited under GenBank accession number MW536483. In this GenBank record and as shown in Table 4, we modified the annotated genome of HHV-6B to include mutations and new splicing regions described in this study. We also confirmed and included new spliced regions found in this study that were also found in previous studies: U19, U79, U83, U91 (18, 29), and U7-U8 and U12-U13, described in Finkel et al. (15). We identified a mutation that introduces a stop codon in U21 (Table 1). The U21 protein is truncated by 125 amino acids compared to the original U21 protein described in AF157706. This mutation was observed in our NGS data on HHV-6B DNA and in all RNA-seq data from the different time points mentioned in this study. However, it is not found in any other HHV-6B sequences in the database. As mentioned in a recent study on HSV-1 (30), we purposely did not rename any ORF in order to avoid causing confusion with previous work. Considerable work has been published on HHV-6B over the last 30 years, and this work forms the basis of our current knowledge. Thus, changing the name of an annotated ORF would cause unnecessary confusion in the field. Finkel et al. have chosen to rename the HHV-6B ORF and include their newly found internal ORF (iORF) and upstream ORF (uORF) in the annotation (15). Inclusion of the iORF and uORF in our modified annotated version of the AF157706 genome would be more suitable for comparison with historical nomenclature. Figure 10 summarizes the genomic organization of the HHV-6B Z29 genome.

Our work is not without limitations. Although we are confident that most of the viral transcripts were detected, some regions had limited, if any, coverage in our data set. These regions could be transcriptionally silent regions or express very low levels of transcripts below the limit of detection. Second, differences between our data and those of others could result from the use of different cell lines for infection. HHV-6B transcriptional regulation and patterns may differ slightly depending on the cell lines for infection. Indeed, this observation was made by Greninger et al. for the U79 spliced transcripts (18). Finally, differences in the HHV-6B viral strains used, or the number of times the virus was passaged (and in which cell type), may also have had an impact on the overall results.

In conclusion, our work provides an up-to-date analysis of the HHV-6B Z29 transcriptome that complements the work of Finkel et al. (15). The data generated should prove useful to generate a better understanding of the complex and highly regulated life cycle of herpesviruses.

MATERIALS AND METHODS

Cell lines and virus. Molt-3 cells (CRL-1552; ATCC) were cultured in RPMI 1640 (Corning Cellgro, Manassas, VA, USA) supplemented with 10% fetal bovine serum (Corning Cellgro), HEPES, and Plasmocin at 5 μg/ml (InvivoGen, San Diego, CA, USA). HHV-6B (Z29 strain) was propagated in Molt-3 cells, as previously described (31).

Infection. Seventy million Molt-3 cells were infected with HHV-6B at a multiplicity of infection (MOI) of 0.5 for 4 h in 4 ml of supplemented RPMI 1640. Cells were then washed twice with 1× phosphate-buffered saline (PBS). DNA was extracted from 1,000,000 cells, and the remaining cells were resuspended at 500,000 cells/ml in supplemented RPMI 1640. Ten million cells were harvested at 6, 9, 12, 24, 48, and 72 h postinfection, and the cell pellets were stored at −80°C until extraction. DNA was extracted from 1,000,000 cells at 72 h postinfection. Ten million Molt-3 cells were incubated in the presence of 10 μg/ml cycloheximide (CHX) (MilliporeSigma Canada, Oakville, ON, Canada) for 30 min before HHV-6B infection at an MOI of 0.5. Cells were maintained in 600 μl for 4 h, washed twice with 1× PBS, and resuspended at
**TABLE 4** Comparison between AF157706 and MW536483 considering the results obtained in this study and previous studies

| ORF   | Strand orientation | AF157706 | MW536483 | Comment |
|-------|--------------------|----------|----------|---------|
|       |                    | Start    | Stop     | Start   | Stop     |
| DR1-L | +                  | 583      | 841      | 583     | 841      |
|       |                    | 955      | 955      | 2975    | 2975     |
| DR3-L | –                  | 2723     | 3325     | 2723    | 3325     |
| B1-L  | +                  | 3022     | 3501     | 3022    | 3501     |
| B2-L  | +                  | 3536     | 3775     | 3536    | 3775     |
| DR6-L | +                  | 5027     | 5330     | 5027    | 5330     |
|       |                    | 6329     | 7203     | 6330    | 7204     |
| B3-L  | +                  | 7349     | 7528     | 7350    | 7529     |
| B4    | –                  | 8911     | 9492     | 8912    | 9493     |
| B5    | +                  | 9522     | 9761     | 9523    | 9762     |
| U2    | –                  | 9624     | 10715    | 9625    | 10716    |
| U3    | –                  | 11045    | 12205    | 11046   | 12206    |
| U4    | –                  | 12433    | 14041    | 12434   | 14041    |
| U5    | –                  | 14159    | 15800    | 14160   | 15801    |
|       |                    | 15890    | 16959    | 15891   | 16866    |
|       |                    |          |          |         |          |
| U6    | +                  | 15603    | 15809    | 15604   | 15810    |
| U8    | –                  | 16963    | 18198    |         |          |
| U9    | –                  | 18179    | 18493    | 18180   | 18494    |
| U10   | +                  | 18543    | 20054    | 18544   | 20055    |
| U11   | –                  | 19958    | 22534    | 19959   | 22535    |
| U12   | +                  |          |          |         |          |
|       |                    | 22636    | 22668    | 22618   | 22669    |
|       |                    | 22746    | 23330    | 22747   | 23331    |
| U13   | +                  | <20801   | 20851    |         |          |
|       |                    | 22618    | 22669    | Part of U7 |
|       |                    |          |          |         |          |
| U14   | +                  | 23856    | 24179    | 23854   | 24180    |
| U15   | –                  | 24277    | 26109    | 24278   | 26110    |
| U17   | –                  | 26143    | 26328    | 26144   | 26329    |
| U18   | –                  | 26500    | 26572    | 26501   | 26573    |
| U19   | –                  | 26732    | 27048    | 26733   | 27049    |
| U20   | –                  | 27330    | 28105    | 27331   | 28106    |
| U21   | –                  | 28193    | 28421    | 28194   | 28422    |
| U22   | –                  | 29601    | 30485    | 29602   | 30486    |
| U23   | –                  |          |          |         |          |
| U24   | –                  | 30499    | 30541    |         |          |
| U25   | –                  |          |          |         |          |
| U26   | –                  | 30751    | 31920    | 30756   | 31921    |
| U27   | –                  | 31245    | 33449    | 31246   | 33450    |
| U28   | –                  | 33452    | 34954    | 33828   | 34955    |
| U29   | –                  | 34851    | 35459    | 34852   | 35460    |
| U30   | –                  | 35487    | 36386    | 35488   | 36387    |
| U31   | +                  | 36511    | 36777    | 36512   | 36778    |
| U32   | –                  | 36796    | 36969    | 36797   | 36970    |
| U33   | –                  | 36986    | 37936    | 36987   | 37937    |
| U34   | –                  | 38044    | 38931    | 38045   | 38932    |
| U35   | –                  | 38919    | 40019    | 38920   | 40020    |
| U36   | –                  | 40135    | 42549    | 40136   | 42550    |
| U37   | –                  | 42572    | 42471    | 42573   | 43472    |
| U38   | –                  | 42999    | 46247    | 43000   | 46248    |
| U39   | –                  | 46265    | 52498    | 46266   | 52499    |
| U40   | –                  | 52572    | 52841    | 52573   | 52842    |
| U41   | –                  | 52843    | 54255    | 52844   | 54256    |
| U42   | –                  | 54206    | 55036    | 54207   | 55037    |
| U43   | –                  | 55053    | 55373    | 55054   | 55374    |
| U44   | +                  | 55372    | 56826    | 55373   | 56827    |
| U45   | –                  | 56830    | 57624    | 56831   | 57625    |
| U46   | –                  | 57670    | 60708    | 57671   | 60709    |
| U47   | –                  | 60708    | 63200    | 60709   | 63201    |
| U48   | –                  | 63154    | 65334    | 63155   | 65335    |
| U49   | –                  | 65342    | 68740    | 65343   | 68741    |
| U50   | –                  | 70103    | 71653    | 70104   | 71654    |
| U51   | –                  | 71878    | 74460    | 71879   | 74461    |

(Continued on next page)
| ORF | Strand orientation | ORF Strand orientation | ORF Strand orientation |
|-----|---------------------|------------------------|------------------------|
| U44 | +                   | 74501 75196 74502 75129 | 74501 75196 74502 75129 |
| U45 | –                   | 75143 76273 75144 76274 | 75143 76273 75144 76274 |
| U46 | +                   | 76047 76071 76048 76071 | 76047 76071 76048 76071 |
| U47 | –                   | 75143 76273 75144 76274 | 75143 76273 75144 76274 |
| U48 | –                   | 79265 81349 79266 81350 | 79265 81349 79266 81350 |
| U49 | +                   | 81508 82266 81509 82267 | 81508 82266 81509 82267 |
| U50 | +                   | 82043 83710 82044 83711 | 82043 83710 82044 83711 |
| U51 | +                   | 83808 84713 83809 84714 | 83808 84713 83809 84714 |
| U52 | –                   | 84732 85508 84733 85509 | 84732 85508 84733 85509 |
| U53 | +                   | 85515 87101 85516 87102 | 85515 87101 85516 87102 |
| U54 | –                   | 87336 88715 87337 88716 | 87336 88715 87337 88716 |
| U55 | +                   | 91750 92876 91751 92877 | 91750 92876 91751 92877 |
| U56 | –                   | 91164 95201 91165 95202 | 91164 95201 91165 95202 |
| U57 | –                   | 92513 97531 92514 97532 | 92513 97531 92514 97532 |
| U58 | +                   | 97528 98580 97529 98581 | 97528 98580 97529 98581 |
| U59 | +                   | 98577 99704 98578 99705 | 98577 99704 98578 99705 |
| U60 | –                   | 102912 103784 102913 103785 | 102912 103784 102913 103785 |
| U61 | +                   | 103756 104817 103757 104818 | 103756 104817 103757 104818 |
| U62 | +                   | 104817 105161 104818 105162 | 104817 105161 104818 105162 |
| U63 | +                   | 105164 106855 105165 106856 | 105164 106855 105165 106856 |
| U64 | +                   | 108593 109627 108594 109628 | 108593 109627 108594 109628 |
| U65 | +                   | 121328 121376 121329 121377 | 121328 121376 121329 121377 |
| U66 | +                   | 121328 121376 121329 121377 | 121328 121376 121329 121377 |
| U67 | +                   | 121328 121376 121329 121377 | 121328 121376 121329 121377 |
| U68 | +                   | 121328 121376 121329 121377 | 121328 121376 121329 121377 |
| U69 | +                   | 121328 121376 121329 121377 | 121328 121376 121329 121377 |
| U70 | +                   | 121328 121376 121329 121377 | 121328 121376 121329 121377 |
| U71 | +                   | 121328 121376 121329 121377 | 121328 121376 121329 121377 |
| U72 | +                   | 121328 121376 121329 121377 | 121328 121376 121329 121377 |
| U73 | +                   | 121328 121376 121329 121377 | 121328 121376 121329 121377 |
| U74 | +                   | 121328 121376 121329 121377 | 121328 121376 121329 121377 |
| U75 | +                   | 121328 121376 121329 121377 | 121328 121376 121329 121377 |
| U76 | +                   | 121328 121376 121329 121377 | 121328 121376 121329 121377 |
| U77 | +                   | 121328 121376 121329 121377 | 121328 121376 121329 121377 |
| B6  | +                   | 121328 121376 121329 121377 | 121328 121376 121329 121377 |
| B7  | +                   | 121328 121376 121329 121377 | 121328 121376 121329 121377 |
| U79-1 | +                | 121328 121376 121329 121377 | 121328 121376 121329 121377 |
| U79-2 | +                | 121328 121376 121329 121377 | 121328 121376 121329 121377 |
| U81 | +                   | 121328 121376 121329 121377 | 121328 121376 121329 121377 |
| U82 | +                   | 121328 121376 121329 121377 | 121328 121376 121329 121377 |
| U83 | +                   | 121328 121376 121329 121377 | 121328 121376 121329 121377 |
| U83R| +                   | 121328 121376 121329 121377 | 121328 121376 121329 121377 |
| U84 | +                   | 121328 121376 121329 121377 | 121328 121376 121329 121377 |
| U85 | +                   | 121328 121376 121329 121377 | 121328 121376 121329 121377 |
| U86 | +                   | 121328 121376 121329 121377 | 121328 121376 121329 121377 |

(Continued on next page)
500,000 cells/ml in supplemented RPMI 1640 containing 10 μg/ml CHX. Cells were harvested at 9 h post-infection, and the pellet was stored at −80°C until extraction. Ten million Molt-3 cells were incubated in the presence of 100 μg/ml phosphonoacetic acid (PAA) (MilliporeSigma Canada) for 30 min before HHV-6B infection at an MOI of 0.5. Cells were maintained in 600 μl for 4 h and washed twice with 1× PBS. DNA was extracted from 1,000,000 cells, and the remaining cells were resuspended at 500,000 cells/ml in supplemented RPMI 1640 containing 100 μg/ml PAA. Cells were harvested at 72 h postinfection, and the pellet was kept stored at −80°C until extraction. DNA was extracted from 1,000,000 cells at 72 h postinfection in the presence of PAA. Ten million uninfected Molt-3 cells were also harvested as a negative control.

DNA extraction and ddPCR. DNA was extracted from cells using the QiaAMP DNA blood minikit as described by the manufacturer (Qiagen Inc.) and analyzed by ddPCR as previously described (32). In brief, 10 ng of gDNA from each time point was analyzed using primers and probes designed to detect the HHV-6B U67-U68 gene and the RPP30 reference cellular gene. Data were normalized to the corresponding genome copies of the cellular RPP30 gene and expressed as copies per cell (33).

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FIG 10 HHV-6B genomic organization. The upper section shows the positions of the major repeat elements (DR, R1, R2, and R3), the unique region, and the origin of replication. The ORFs are indicated in the lower section, and their colors reflect their kinetic classes. This figure was modified from the original version initially published by Dominguez et al. (11).
RNA-seq. Libraries were generated for all conditions using the Ion Total RNA-Seq kit v2 (Thermo Fisher Scientific, Ottawa, ON, Canada) by following a standard protocol. Libraries were then sequenced on an Ion Torrent S5 sequencer (Thermo Fisher Scientific).

Sequencing returned 94 million reads with greater than 9.4 million reads per condition. Reads were aligned to the *Homo sapiens* reference genome GRCh38 and to the Z29 strain of HHV-6B (NCBI accession number AF157706). Alignments were performed using Bowtie2 v2.3.4.1, and junction prediction was determined using TopHat v2.1.1. Transcriptional and gene read counts were obtained with CLC Genomics Workbench v9.5.2 (Qagen) using the RNA-seq analysis tool and the genome annotations mentioned above.

**Immunofluorescence.** Immunofluorescence was performed as previously described (34). In brief, HHV-6B-infected Molt-3 cells were deposited on a 10-well microscope slide, dried, and fixed in acetone at −20°C for 10 min. The following primary antibodies were used: rabbit-α-IE1-Alexa-488, mouse-α-P41 (NIH AIDS Reagent Program, Germantown, MD, USA), and mouse-α-DR6/7 (NIH AIDS Reagent Program). Goat α-mouse-Alexa-488 was used as the secondary antibody (Life Technologies Inc., Burlington, ON, Canada). Slides were observed at 63× using a spinning disc confocal microscope (Leica DMI6000B) and analyzed with the software Volocity 5.4.

**RT-PCR.** Ten million Molt-3 cells continuously infected with HHV-6B were harvested. Total RNA was isolated using the standard Qiazol protocol (Qiagen Inc.) and processed for mRNA analysis by reverse transcriptase PCR (RT-PCR). In brief, 1 μg of total RNA was reverse transcribed in a total volume of 20 μl using the Moloney murine leukemia virus reverse transcriptase. One-tenth of the cDNA was used for the amplification of specific splicing junctions, using the primers specified in Table 5. PCR products were amplified, using Q5 high-fidelity DNA polymerase (New England Biolabs Ltd., Whitby, ON, Canada), at 98°C for 1 min, 40 cycles of 10 s at 98°C, 30 s at 60°C, and 3 min at 72°C, and a final elongation step at 72°C for 2 min. PCR products were then run on a 2% agarose gel and visualized using a Gel Doc XR+ System (Bio-Rad Laboratories Canada Inc., Mississauga, ON, Canada).

**Sequencing.** Sanger sequencing and next-generation sequencing were performed at the facility located at the CHU de Québec-Université Laval Research Center.

**Data availability.** The annotated HHV-6B sequence has been deposited under the GenBank accession number MAV536483. Sequencing data files have been deposited under the BioProject accession number PRJNA680783.

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Conceived experiments, A.G., N.M., and L.F. Performed experiments, A.G., W.S, N.M., and L.F. Contribution of key reagents and methodology, A.G., W.S., N.M., A.D., and L.F. Data analysis, A.G., W.S., E.F., and L.F. Writing, A.G. and L.F. Manuscript revision, A.G., W.S., E.F., A.D., N.M., and L.F.

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| Name       | Primer sequence                                                                 | Figure |
|------------|---------------------------------------------------------------------------------|--------|
| U7 Ex2 reverse | 5′-GGCCAGGCGATACAAAT-3′                                                          | 5C     |
| U7 Ex1 forward   | 5′-TTTGGCACTCGCGTTAT-3′                                                          | 5C     |
| U12-U13 Ex2 forward | 5′-GACACTGTCAAGGCTCGTCC-3′                                                      | 6C−E   |
| U12 Ex3 reverse   | 5′-CTTCATAGTCCGCTGTCGCT-3′                                                       | 6C     |
| U12-U13 Ex1 forward | 5′-CGAGCGCTACAGTTTCCAG-3′                                                       | 6G     |
| U12-U13 Ex2 reverse | 5′-CTGGAGACTTACAGGAGAAG-3′                                                      | 6G     |
| U44 Ex1 forward   | 5′-TCTCCATTAGGAGAAGAGGAGTTT-3′                                                   | 7C     |
| U44 Ex2 reverse   | 5′-CCTCTTATACAGGCGCAAATCT-3′                                                     | 7C     |
| U67 Ex1 forward   | 5′-GGATCCGAGAAACTCTGCTGATTG-3′                                                   | 8C     |
| U67 Ex2 reverse   | 5′-CCTCTTACCTGCTGCTGAGATT-3′                                                     | 8C     |
| U69R srcRNA forward | 5′-TCTTCACTAGCTCGGAGATAG-3′                                                     | 9C     |
| U69R srcRNA reverse | 5′-TGAGATTCTCATTATGGTAAAAGC-3′                                                   | 9C     |
| U67-U68 forward   | 5′-TTCGGTGATATGACCTCGTAAGC-3′                                                    | 3A     |
| U67-U68 reverse   | 5′-GATGTCACCTCCTCAATCTTTGAAAT-3′                                                 | 3A     |
| U67-U68 probe     | 5′-FAM-ACATATAT-ZEN-RTCGAAAYYTGACRTACTTCCG-IABkFQ-3′                            | 3A     |
| hRFP30 forward    | 5′-GATTTGACCTCGCGAGG-3′                                                          | 3A     |
| hRFP30 reverse    | 5′-GCCGCGTCTCCTACAAGT-3′                                                         | 3A     |
| hRFP30 Probe      | 5′-HEX-TCTGACCTG-ZEN-AAGGCTCTGCGG-IABkFQ-3′                                     | 3A     |
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