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Episomal replication timing of γ-herpesviruses in latently infected cells

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ABSTRACT

This study addresses the timing of gammaherpesviral episomal DNA replication with respect to the cell cycle. For the first time we analyzed a rhadinovirus, the prototype Herpesvirus saimiri (HVS), and compared it to the lymphocryptovirus Epstein–Barr virus (EBV). Newly synthesized DNA of latently infected B- or T-cells was first BrdU-labeled; then we sorted the cells corresponding to cell cycle phases $G_{0/1}$, $G_{2/M}$, and S (4 fractions S_1–S_4) and performed anti-BrdU chromatin immunoprecipitation. Next, DNA of different viral gene loci was quantitatively detected together with cellular control genes of known replication time. The sensitive technique is further enhanced by an internal coprecipitation standard for increased precision. Both gammaherpesviruses replicated very early in S-phase, together with cellular euchromatin. Our work suggests that early S-phase DNA replication is a general characteristic of episomal herpesviral genomes.

Introduction

The *gammaherpesvirinae* are divided into two genera: the lymphocryptoviruses (γ1-herpesviruses), e.g., Epstein–Barr virus (EBV) and the rhadinoviruses (γ2-herpesviruses), e.g., Kaposi's Sarcoma associated human herpesvirus 8 and Herpesvirus saimiri (HVS).

The lymphocryptovirus EBV has the ability to transform resting B-cells to lymphoblastoid cell lines (LCLs). It utilizes this immortalization and the physiology of normal B-cell differentiation to establish latency in the resting memory B-cell pool. Herpesvirus saimiri (saimiriine herpesvirus type 2, HVS) is endemic but apathogenic for its natural host, the squirrel monkey (*Saimiri sciureus*), a New World primate indigenous to South American rainforests (Melendez et al., 1968). HVS isolates are assigned to three subgroups, A, B, and C, according to pathogenic properties in other primates and sequence variations in a specific region of the viral genome (Desrosiers and Falk, 1982; Medveczky et al., 1984). Virus strains of subgroup C are unique in their ability to transform human T-lymphocytes to stable antigen-independent growth in culture (Biesinger et al., 1992). During latency their genomes persist as non-integrated episomes in the nuclei of the host cells. The latent viral genome is associated with histones, and hetero- and euchromatic regions are distinguishable (Alberter and Ensser, 2007; Stedman et al., 2004, 2008). Actively transcribed genes necessary for latent replication are located within the euchromatic region; genes that are only active during lytic replication and silent during latency are usually heterochromatic. The episomal viral DNA is attached to the cellular DNA by an interplay of cellular factors with viral proteins such as EBNA1 and LANA and is evenly segregated to the daughter cells during cell division (Sugden, 2002; Griffiths and Whitehouse, 2007; Griffiths et al., 2008; Ballestas et al., 1999). The viral DNA is replicated during the S-phase once per cell cycle, synchronously and together with the cellular DNA (Yates and Guan, 1991).

Earlier studies demonstrated that episomal Epstein–Barr virus genome replication occurs in early DNA synthesis (S-)phase (Benz and Strominger, 1975; Hampar et al., 1974). A later study detected late S-phase replication of EBV (Carroll et al., 1991). Zhou et al. (2009) recently concluded that replication of the EBV DNA occurs in mid–late S-phase; in a subset of their experiments they have used a method very similar to the one by Carroll et al. (1991) but refined it by combining it with immunoprecipitation of BrdU-labeled, newly synthesized DNA. Taken together, these variable results were representative only for a single herpesvirus, EBV, and it was often studied in a single cell line. The time at which other viral DNA genomes replicate in the cell cycle S-phase in latently infected cells is largely unknown.

The existing analytical methods require a substantial number of infected cells; therefore, access to stably transformed cell lines is

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usually necessary for such investigations. The lymphotropic gammaherpesviruses provide an ideal opportunity for such studies, as lymphocytic B- or T-cell systems have been established for them. Here, the viral DNA stably persists in high copy numbers forming circular episomes. We therefore decided to study this matter for the first time in a rhadinovirus, HVS, and performed the analysis in parallel for the lymphocryptovirus EBV. This was done repeatedly for several cell lines infected by each virus, including cells derived from the same respective donors, using an accurate method that employs an internal standard to control the immunoprecipitation steps.

Results

The DNA replication of HVS was evaluated along with EBV DNA replication in lymphoblastoid B-cell lines (LCLs); we analyzed two pairs of HVS-transformed T-cell lines and EBV-transformed LCL that

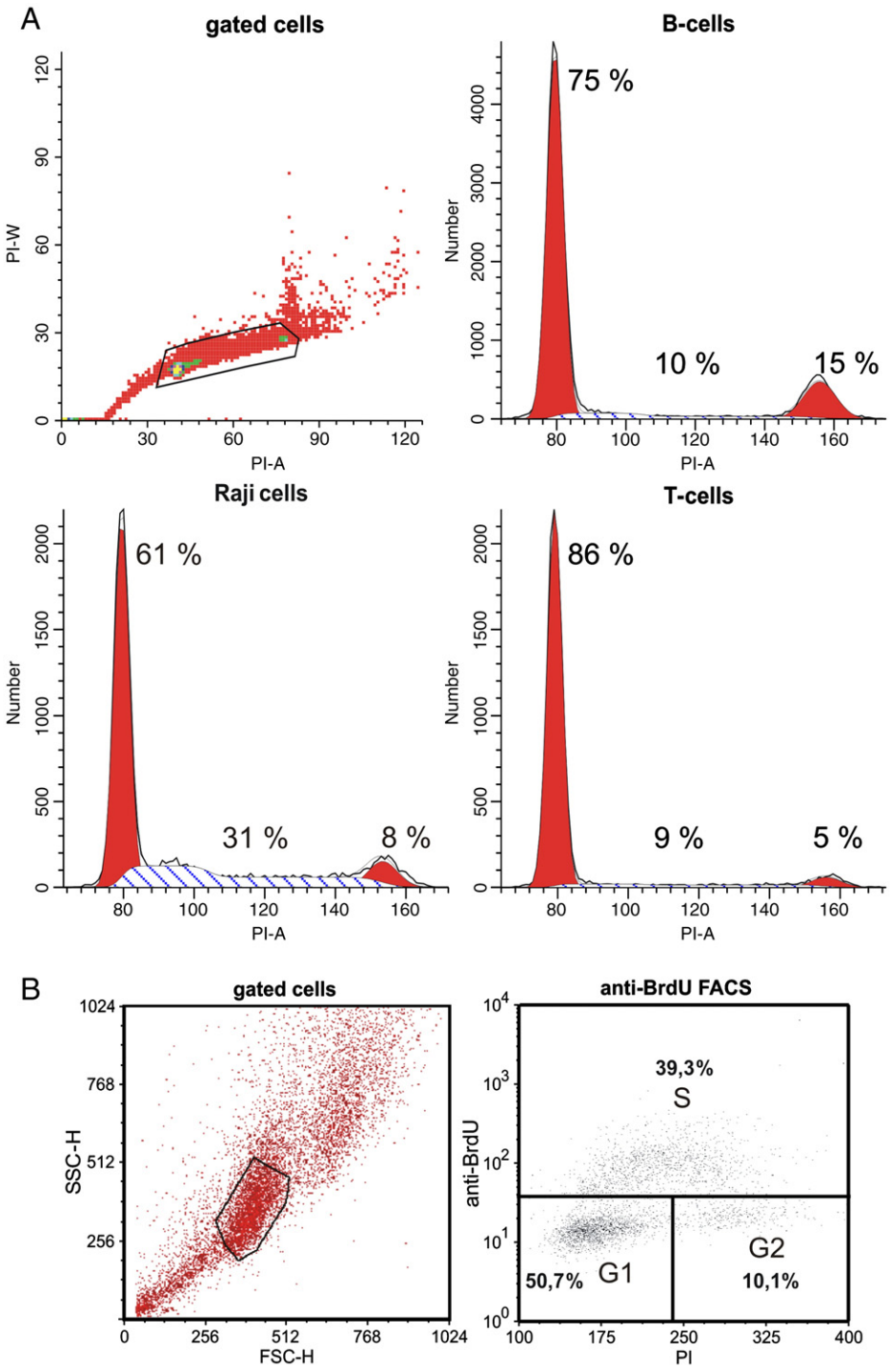


Fig. 1. Cell cycle analysis. (A) Selection of single cells for the flow cytometry analysis of DNA content (upper left: two-dimensional diagram comparing pulse area and width after PI staining). The histograms show DNA content and the according proportion of cells. The results confirm that the faster growing Raji cell line had more cells in S-phase than the EBV-transformed LCLs and the even slower proliferating HVS-transformed T-lymphocytes. The Raji cells have 31%, the LCLs have 10%, and the T-cells have 9% S-phase cells. (B) Anti-BrdU staining of Raji cells that were pulse labeled with BrdU for 1 h. (Left) Selection of cells. (Right) Dot plot of the BrdU-specific signal against the cellular DNA content. The population on the lower left is the cells in G1, on the lower right, cells in G2, and the cells in the arc in the upper half are S-phase cells positively stained for BrdU (approx. 39% of gated cells). The majority of S-phase cells successfully incorporated BrdU.

were established from the same respective two anonymous donors. In addition, the HVS-transformed T-cell line 1810 (Heck et al., 2005) and the Burkitt lymphoma-derived cell line Raji, which was also used in all previous studies, were analyzed.

The growth characteristics and the fraction of cells in S-phase of each analyzed cell line was determined by flow cytometric cell cycle analysis; this information was used to estimate the required number of total cells for the sorting into fractions. The Raji cell line replicated faster than EBV-transformed LCLs; both grew faster than HVS-transformed T-lymphocytes. Raji cells had 31%, LCLs 10%, and HVS-transformed T-cells 9% of cells in S-phase, respectively (Fig. 1A).

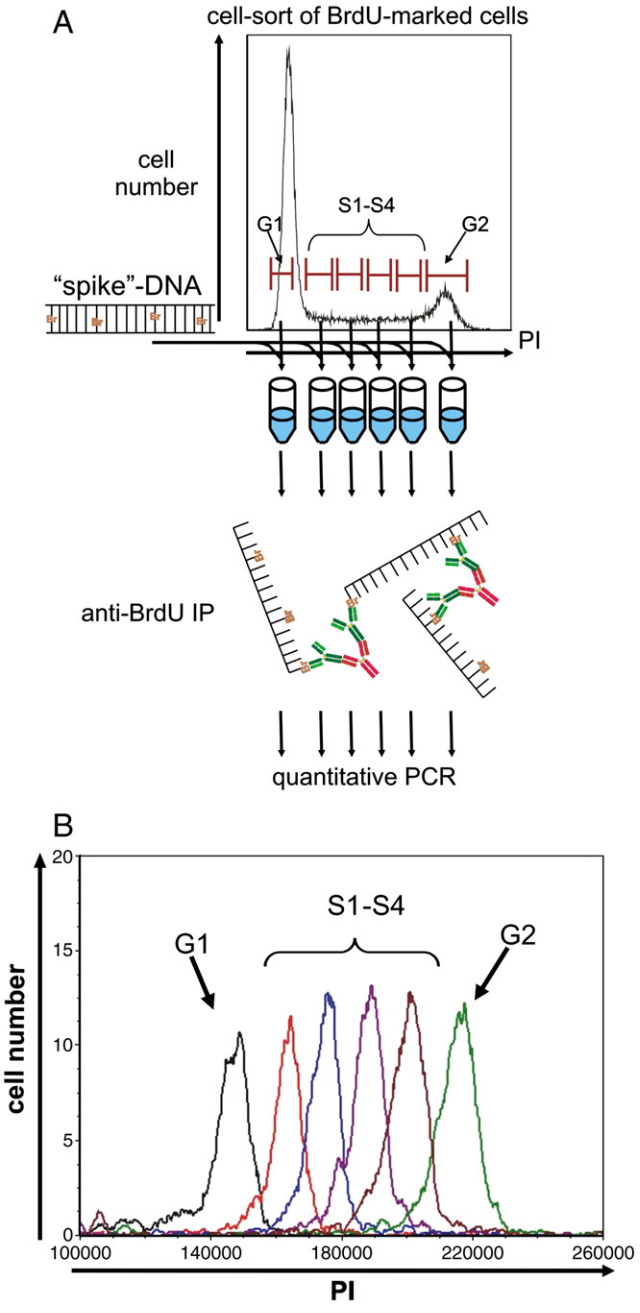


Fig. 2. Experimental procedure. (A) Schematic overview. BrdU is incorporated only into newly synthesized DNA. Equal numbers of BrdU pulse-labeled cells were sorted into six different cell cycle fractions (G1, S1–S4, G2) according to their DNA content. The BrdU labeled internal standard DNA ("spike"-DNA) was added to each sorted fraction in equal amounts. The BrdU-labeled DNA was isolated by anti-BrdU immunoprecipitation and the amount of precipitated DNA was analyzed by quantitative SYBR-Green PCR. (B) Reanalysis of sorted cells. The six sorted cell cycle fractions (G1, S1–S4, G2) were restained (PI) and reanalyzed immediately after sorting. Six distinct and accurately separated populations with minimal overlap can be observed.

The fluorescence cell sorting-based technique that we adapted to analyze replication timing of viral DNA was initially developed for cellular genes (Gilbert, 1986; Azuara et al., 2003; Azuara, 2006). For cellular DNA, it has revealed that densely packed heterochromatin replicated later than loosely packed euchromatin during the synthesis phase. Pulse labeling of newly synthesized DNA with 5′-bromodeoxyuridine (BrdU) was followed by subfractionation of cell cycle phases according to cellular DNA content by fluorescence-activated cell sorting; increased precision is achieved by using an internal standard added during the lysis step to control the subsequent quantitative BrdU–chromatin immunoprecipitation (IP) analysis. In our case we used an internal standard DNA fragment ("spike") corresponding to pos. 8061–8967 of the murine gammaherpesvirus 68 genome, which was PCR amplified in the presence of BrdUTP. Using this procedure, an accurate determination of episomal replication timing was possible.

All cell lines were pulse labeled with BrdU for 1 h. The cellular DNA was quantified by propidium iodide staining and incorporated BrdU was detected by intracellular flow cytometry with APC-conjugated anti-BrdU monoclonal antibody, as shown for Raji cells (Fig. 1B). As expected, proliferating S-phase cells showed an increase in BrdU content, while cells in G0/1 and G2/M were BrdU negative.

A schematic overview of the experimental procedure and the placement of sorting gates used to obtain the six different cell cycle fractions is shown in Fig. 2A. Three fractions of 100,000 cells each were collected in parallel. In order to minimize cross contamination with cells of other fractions, G1, S2, S4 and S1, S3, G2 fractions were sorted at the same time. Independent sorting experiments were done on a FACS-Vantage cell sorter (four experiments) and a MoFlo cell sorter (eight experiments); equipment-related differences were neither observed in the separation of the cell cycle fractions, nor in the results. In Fig. 2B, an overlay of the reanalyzed sorted fractions obtained with a MoFlo cell sorter is shown. This was done to determine the accuracy of the sorter and the purity of sorted fractions. These results show that all six samples were only minimally overlapping into each other, were well separated and that cross contamination between adjacent fractions was minimal.

In order to obtain a rough estimation in which sorted cell cycle fraction of the viral DNA is replicated, we performed a quantitative SYBR-Green PCR with sorted cells but without anti-BrdU IP. The replication times of the cellular control genes and of two loci within the EBV genome (oriP and BVRF1) are shown in Fig. 3. The ordinate indicates the "relative genome copies" measured for each cell cycle fraction. The cellular control genes copy number doubled either in an early (CD45: S1 fraction) or late (alpha-amylase: S4 fraction) S-phase fraction. The viral oriP and BVRF1 DNA is replicated very early during S-phase (S1 fraction).

Quantitative detection of replicated DNA loci after enrichment by anti-BrdU immunoprecipitation shows early S-phase replication of gammaherpesviruses

The abundance of precipitated cellular and viral DNA was determined by quantitative locus-specific SYBR-Green PCR and is shown as the percentage of the total signal abundance of each sorted cell cycle fraction (G1, S1–S4, G2). Fig. 4 shows the genomic localization of the analyzed loci. The results of the replication timing of cellular control genes are in agreement with the data published previously. The control gene for early replication CD45 shows the peak signal in the very earliest S-phase fraction (S1). The controls for late replication timing have the strongest percentage signal in either S3 (HBD) or S4 (Amy1; Figs. 5A and B). These data confirm that the method allowed a clean separation of genes with different replication timing also in our experimental platform. Unspecific binding and precipitation of anti-BrdU antibody to unmarked DNA could be

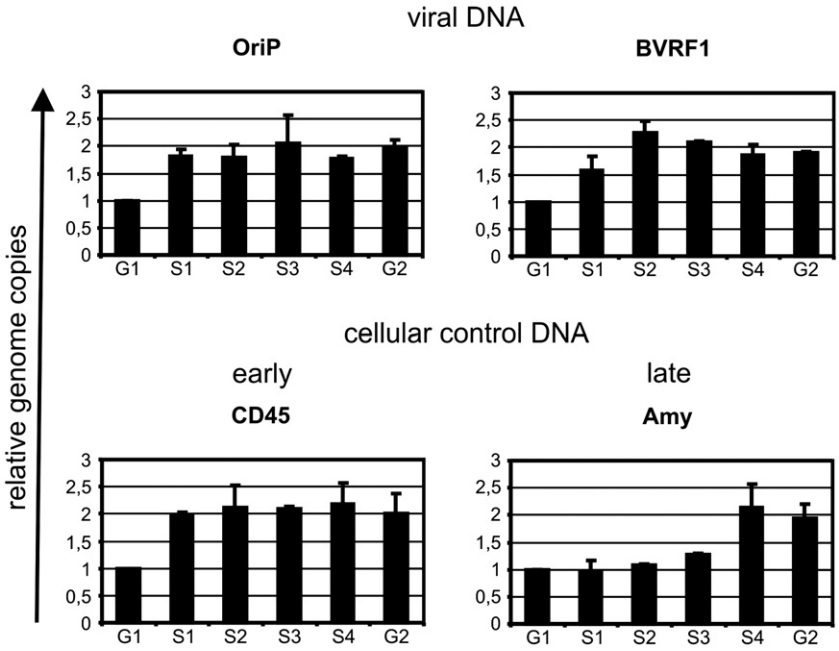


Fig. 3. Duplication of viral and cellular loci during cell cycle. Relative genome copies from sorted Raji cell cycle fractions determined by quantitative SYBR-Green PCR, experiment without immunoprecipitation of newly synthesized DNA. (Upper panels) Duplication of viral DNA occurs in S1 (OriP) and S1/S2 (BVRF1). (Lower panels) Results for the cellular control DNA loci with known replication timing: duplication of the CD45 (early) gene locus in S1, duplication of α-amylase (late) locus in the S4 phase.

excluded through the comparison of anti-BrdU immunoprecipitation of labeled versus unlabeled cells. It could be shown that the precipitated signal from unlabeled cells was less than 2% of labeled DNA (data not shown).

The evaluation of the quantitative PCRs for HVS gene loci (Fig. 5A) generally defines a very early replication timing for viral episomes. The lytic genes that were analyzed code for the major capsid protein (orf25, late) and for the R-transactivator of transcription (orf50, early), homologue to EBV Rta. The latent genes tested encode the StpC/Tip protein (orf1) and the orf73 homologue of KSHV LANA (orf73). In addition to those genes we analyzed the repetitive sequences flanking the viral genome (H-DNA). H-DNA and latent genes in euchromatic regions of the viral episome show their main peak of relative signal abundance in the S1 fraction, the lytic genes located in the heterochromatic region of the episome (Alberter and Ensser, 2007) also show discrete signals in the S2 fractions, suggesting a small delay in replication time compared to the latent genes.

The results of the loci analyzed throughout the viral genome show a very early replication timing of EBV episomes (Fig. 5B). All three analyzed cell lines were consistent in their results for cellular and viral replication timing in independently performed sets of experiments. The main peak of the relative signal abundance of viral DNA could always be observed in the earliest of the sorted S-phase fractions, S1. A distinct main peak in S1 for the loci within and surrounding the origin of persistence ("oriP", "5′oriP", "3′oriP"), the latent membrane protein 1 ("LMP1") gene and the repetitive sequences in the EBV genome ("repeat") can be observed. As in the case of HVS, a small delay of viral DNA replication timing is observable within most loci of lytic genes that are assumed to be packed in heterochromatin. BVRF1 codes for a DNA packaging tegument protein, BRLF1 codes for the BamHI R fragment transactivator of transcription (Rta), in both cases a distinct signal above the level of signal noise can be detected in the S2 fraction.

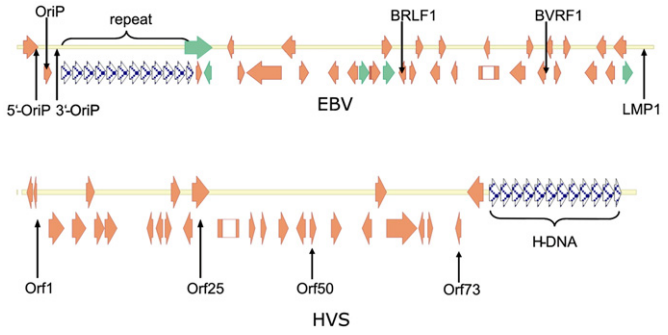


Fig. 4. Localization of analyzed loci in EBV and HVS genomes. In case of EBV, three loci inside and surrounding the OriP and in the repeat region were analyzed. In addition to those three genes we selected LMP1 (actively transcribed during latency), BVRF1, and BRLF1 (lytic genes, silent during latency). In case of HVS, the repetitive H-DNA, two lytic genes in the heterochromatin (orf25 and orf50, silent during latency) and two genes in the euchromatin (orf1 and orf73, active during latency) were analyzed.

Discussion

The variable results on EBV and the paucity of data regarding further gammaherpesviruses prompted us to compare the episomal DNA replication timing in two gammaherpesviruses of different genera in more depth.

In case of HVS, the results for the genes in the euchromatic region of the viral genome all show the main signal in the very first of the sorted S-phase fractions (S1). The genes in the heterochromatic region (orf50 and orf25) are replicated slightly later than the genes in the euchromatin, evident from a distinct shift of the signal toward the S2 phase. The DNA synthesis phase of human cells commonly lasts about 6 to 7 h (Cameron and Greulich, 1963). Therefore, each of the four S-phase fraction should represent 1.5- to 1.75-h duration.

In their elegant analysis, Norio and Schildkraut (2001, 2004) have shown that replication forks move at a rate of ~1 kb/min in the Raji EBV genome, without dependence on transcription in the region, and that the time required to replicate a genomic region is a function of the number of replication origins within the region. They detected regions within the episomal DNA that usually replicate first and regions that replicate last, but those regions differed between the analyzed EBV strains (Raji and Mutu I). Based on the small size of the HVS episomes (approx. 150–170 kb), and in analogy to EBV (Norio and Schildkraut, 2004), the estimated replication by cellular DNA

polymerases would be completed within 20–40 min. Although we did not directly address the formation or movement of viral replication origins, the slight retardation in the replication of the HVS lytic gene loci could be explained by a delayed formation of origins in viral heterochromatin compared to the open euchromatin, rather than by the simply larger distance from the start of replication and the loci that have already been replicated during S1 phase. This is compatible with the observation that replication timing is strongly coupled to histone modifications: DNA packed in acetylated histones is replicated early in S-phase and vice versa the heterochromatin later (Cedar and Bergman, 2009; Lande-Diner et al., 2009).

The results of the experiments in Fig. 5B show an early replication of episomal EBV DNA during the synthesis phase of the cell cycle in latently infected cells. Comparable to HVS lytic genes, the BVRF1 and BRLF1 loci, which are not expressed during latency, show a tendency toward a delayed replication timing compared to actively transcribed lytic genes. In case of EBV, there are no genome-wide analyses on the eu- or heterochromatic state of different genes during latency available. However, the delayed replication could indicate that BVRF1 and BRLF1 are rather heterochromatic, comparable to orf50 and orf25 of HVS.

In case of EBV, publications about its replication timing are at variance. Earlier studies have determined the timing of episomal Epstein–Barr virus genome replication in early S-phase in Raji cells (Benz and Strominger, 1975; Hampar et al., 1974). These direct studies of gammaherpesviral DNA replication timing were appropriate and state of the art at that time. They have applied a combination of partially synchronized cells released from cell cycle arrest by double thymidine block and metabolic labeling by an [H^3]dT pulse. The timely resolution was limited by only partial synchronization of cells, by the variable onset of replication after release from the block, and by the detection procedure involving hybridization and autoradiography.

The later data by Carroll et al. (1991) and the recent work by Zhou et al. (2009) hinted at a delayed replication of viral episomes during S-phase. It remains difficult to explain these variable published results for EBV, especially the recent publication from Zhou et al., who used a relatively similar approach to ours in a subset of their experiments. They used zonal elutriation to obtain S-phase fractions, a method that can be used to obtain a large number of cells enriched in size and specific DNA content, but where substantial contamination with cells from other fractions remains. Less probable reasons might be the use of different primer pairs for the cellular control PCR, or impurities in FACS sorting, that can only be judged when a reanalysis of the sorted fractions is shown (Fig. 1B).

The cell sorting, DNA-content-based fractionation method that we applied does not require survival of the cell until analysis. This is ideal for this purpose, as it can more accurately exclude aggregates and apoptotic cells. Therefore it delivers cell fractions of higher purity than S-phase fractions that are be obtained by separation techniques based on cell density or size alone, such as zonal elutriation.

Reactivation of viral lytic replication occurs in S-phase and may be a theoretical concern in the analysis of EBV, since the elevated viral DNA content of few reactivating cells could result in a signal comparable to many cells harboring latently replicating virus. However, the defective EBV genome in Raji (Polack et al., 1984; Fresen et al., 1978) cannot reactivate, and we did not stimulate the other EBV LCL. HVS is strictly latent in human T-cells. Even stimulation with agents that commonly lead to herpesviral reactivation does not result in release of viral particles (Fickenscher et al., 1996).

Our work is the first analysis in a rhadinovirus, and we compared the replication timing for two closely related gammaherpesviruses. HVS and EBV were repeatedly analyzed in three different cell lines for each virus, respectively, and two pairs of B- and T-cells were from identical donors. Each of our analyses was done in at least two independent sorting experiments and data of the reanalysis of the cell sort show a high accuracy of the sorting equipment and minimal overlap between the sorted cell cycle fractions. The use of an internal standard that is coprecipitated during the BrdU–ChIP experiment further enhanced the quality of the data in our experiments by providing a measure and control for the IP efficiency. Furthermore, in our experiments DNA synthesis as evident by BrdU incorporation could only be observed in the synthesis phase. The cellular controls always showed a time of replication in the same respective fraction across the various cell lines and experiments, highlighting the stability of the method applied. The robustness of the established method provides an opportunity to determine the replication timing of further viruses as long as stable cell systems are available.

In summary, the results of our study hint at early S-phase replication of the gammaherpesviruses EBV and HVS in B- and T-cells, respectively. The early viral DNA genome replication in latently infected cells is a prerequisite for the EBNA1- and LANA homologue-mediated segregation of viral episomes to daughter cells and has to occur before the separation of host cell chromatides begins. In the future, the replication timing of latent and lytic genes can be analyzed during the progression of the latent to the lytic states in the viral replication cycle, e.g., after induction by substances that modify epigenetic information, or under the influence of antiviral drugs.

Material and methods

Cell lines

Herpesvirus saimiri-transformed T-cell lines and Epstein–Barr virus-transformed lymphoblastoid B-cell lines were established from the same respective two anonymous donors as described previously (Heck et al., 2006; Nilsson et al., 1971; Miller and Lipman, 1973). In addition, the HVS-transformed T-cell line 1810 (Heck et al., 2005) and the Burkitt lymphoma-derived cell line Raji (ATCC CCL-86) were analyzed. Human T-lymphocyte cultures were grown in RPMI-1640 (Invitrogen) and Panserin 401 medium mixed at a ratio of 1:1 supplemented with 10% irradiated FCS (Pan Biotech, Aidenbach, Germany), 10 U/ml recombinant human interleukin-2 (Roche Applied Science), glutamine (350 μg/ml), and gentamycin (100 μg/ml). B-cell lines were maintained in RPMI-1640 (Invitrogen) supplemented with 10% irradiated FCS, glutamine (350 μg/ml), and gentamycin (100 μg/ml).

BrdU internal standard DNA ("spike"-DNA)

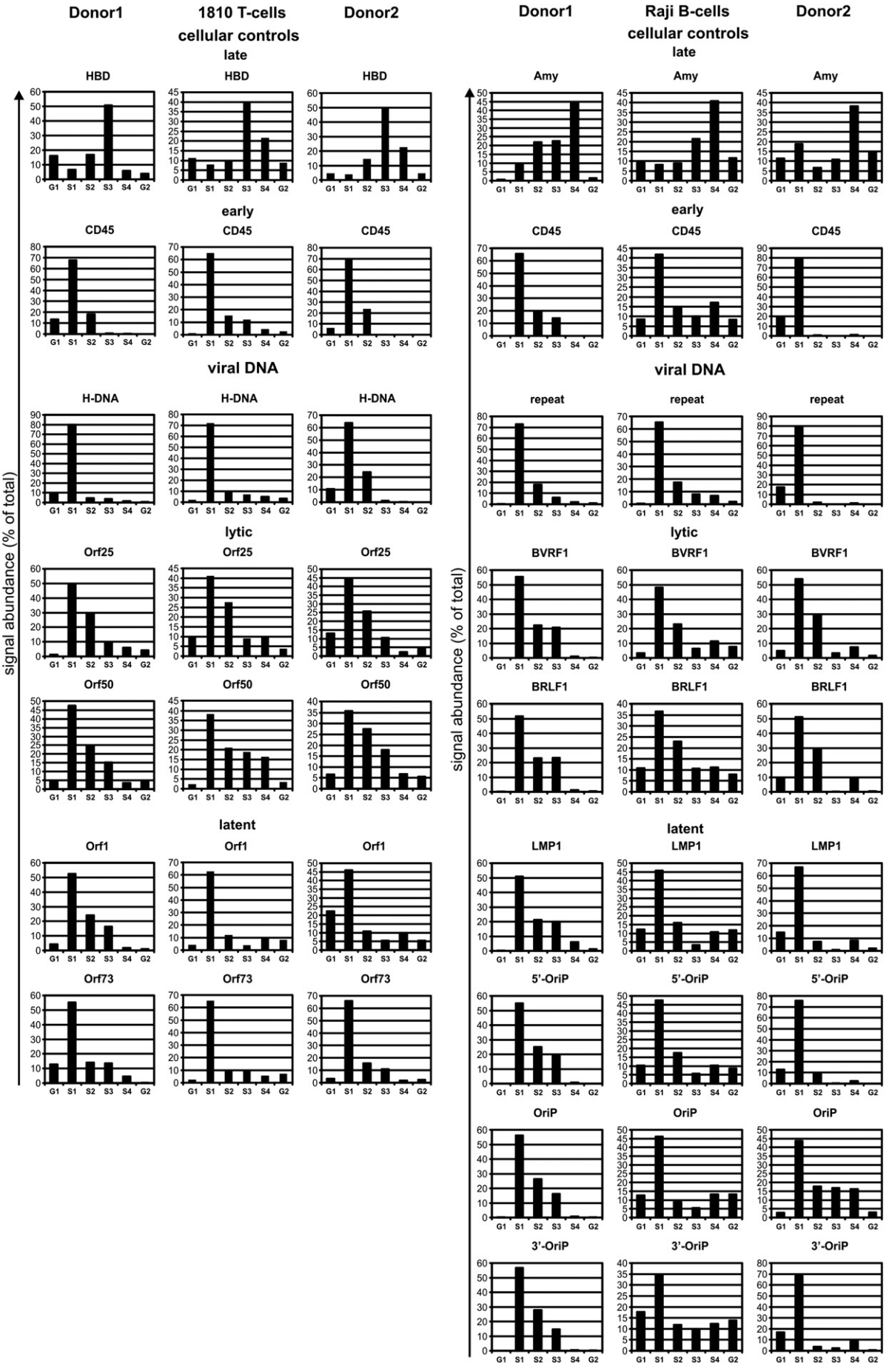
A fragment corresponding to pos. 8061–8967 of the murine gammaherpesvirus 68 (MHV68) genome was PCR amplified in the presence of 200 nM dNTP and 20 nM BrdUTP with the oligonucleotide primers mhvM2a GGTGATGGGACAGACGGGAACTAC and mhvB1 CTGGTGCCACTCAGAATGTTCAGTG from plasmid pMHVShuttle (kindly provided by Heiko Adler, Munich). The BrdU-labeled PCR amplicons were purified by extraction with phenol/chloroform/isoamylethanol (25:24:1) and precipitated with ethanol. BrdU incorporation was verified by electrophoresis and anti-BrdU dot-blot analysis.

Antibodies and antisera

The antibodies and antisera used are given as follows: Rabbit polyclonal anti-mouse IgG (H+L; AffiniPure, Jackson ImmunoResearch (IR), Baltimore), mouse monoclonal anti-BrdU clone BU-33 (Sigma-Aldrich), mouse monoclonal anti-BrdU-allophycocyanine (APC BrdU Flow Kit, BD Pharmingen), swine polyclonal anti-mouse horseradish peroxidase (Dako).

Anti-BrdU flow cytometry

Two million cells were pulse labeled with BrdU for 45 min. The cells were incubated in 500 μl ethanol (95%, ice cooled) on ice for 30


Donor1
1810 T-cells
Donor2
Donor1
Raji B-cells
Donor2
cellular controls
late
HBD
Amy
early
CD45
viral DNA
H-DNA
repeat
lytic
Orf25
BVRF1
Orf50
BRLF1
latent
Orf1
LMP1
Orf73
5'-OriP
OriP
3'-OriP
signal abundance (% of total)
G1 S1 S2 S3 S4 G2

min and in 500 µl 1% paraformaldehyde and 0.01% Tween-20 at room temperature for 30 min. The cellular DNA was partially digested in 200 µl PBS with DNase (AppliChem, final concentration of 50 µg/ml) at 37 °C for 60 min in order to make the BrdU epitope accessible for the antibody. The cells were marked in 200 µl PBS with a 1:4000 dilution of an APC-labeled anti-BrdU antibody (APC BrdU Flow Kit, BD Biosciences). After incubation in 200 µl staining buffer (40 mM Tris–HCl at pH 7.4, 0.8% NaCl, and 21 mM $MgCl_2$) supplemented with 0.05% NP-40, propidium iodide (PI, 50 µg/ml), and RNase A (1 mg/ml) for 30 min, the cells were analyzed at a FACScalibur flow cytometer (BD Biosciences) and evaluated with FCS Express V3 (De Novo Software, Los Angeles, CA).

Isolation of BrdU-labeled DNA by immunoprecipitation

BrdU-labeled DNA was isolated as described (Hansen et al., 1993). Equal amounts of BrdU-labeled internal standard DNA were added to each fraction before phenol–chloroform extraction and ethanol precipitation. Precipitated DNA was dissolved in 500 µl Tris-EDTA, mixed with 0.2 mg of denatured, sheared salmon sperm DNA, sonicated to generate fragments of an average size of 700 bp (range 0.25–2 kb as determined by gel electrophoresis), heat-denatured, and cooled on ice. Samples were adjusted to 10 mM sodium phosphate at pH 7, 0.14 M NaCl and 0.05% Triton X-100 before incubation with 80 µl of mouse anti-BrdU antibody (25 µg/ml; BD Biosciences) for 20 min at room temperature followed by addition of excess secondary antibody (35 µg of rabbit anti-mouse IgG; Jackson IR) for 20 min at room temperature. DNA–protein complexes were collected by centrifugation, washed, resuspended in 200 µl lysis buffer (50 mM Tris–HCl at pH 8, 10 mM EDTA, 0.5% SDS, and 0.25 mg/ml proteinase K), and incubated overnight at 37 °C. Twenty micrograms of yeast tRNA were added and DNA was extracted with phenol–chloroform and precipitated with ethanol. Recovered BrdU-labeled DNA was resuspended in Tris-EDTA at a concentration of 1000 input cell equivalents per microliter.

Cell cycle flow cytometry

One million cells were incubated in staining buffer (40 mM Tris at pH 7.4, 0.8% NaCl, and 21 mM $MgCl_2$) supplemented with 0.05% NP-40, PI (50 µg/ml), and RNase A (1 mg/ml) for 30 min. The cells were analyzed at a FACScalibur flow cytometer (BD Biosciences). In all FACS experiments, living cells were gated according to cell size and granularity in a forward/sideward scatter two-dimensional plot. Single cells were then distinguished from doublets by comparing signal pulse area and pulse width of PI-fluorescence intensity in a two-dimensional plot and gated accordingly to exclude doublets, which would otherwise contaminate the G2 fraction (Fig. 1A, upper left; Shapiro, 2003). The resulting data were evaluated with ModFit LT V3.1 (Verity Software House, Topsham, ME).

BrdU labeling and cell cycle fractionation by flow cytometry

BrdU labeling was done by incubating the cells in culture with 50 µM 5′-bromodeoxyuridine for 60 min. The cells were then fixed with 70% ice-cold ethanol for 30 min. Right before sorting, BrdU-labeled cells were resuspended in staining buffer (40 mM Tris at pH 7.4, 0.8% NaCl, and 21 mM $MgCl_2$) supplemented with 0.05% NP-40, PI (50 µg/ml), and RNase A (1 mg/ml) for 30 min. Equal numbers of cells (between 50,000 and 100,000) were collected for each of six fractions (G1, S1, S2, S3, S4, and G2; Fig. 1A) into lysis buffer (1 M NaCl, 10 mM EDTA, 50 mM Tris–HCl at pH 8, 0.5% SDS, 0.2 mg/ml proteinase K, and 0.25 mg/ml denatured, sheared salmon sperm DNA). Fractions were incubated for 2 h at 50 °C and stored at -20 °C.

PCR amplification

The following oligonucleotide sequences (5′–>3′) were used as primers for PCR amplification. Cellular controls: CD45_r CCATTGACAGAATGTTCTGG, CD45_f AGTGGACAAAGTAAAGCAGG; HBD_r GGAGGAAGCTATTTCTTGGAGCAGG, HBDf AAAGCAAGGGAACCGTACAAGGC; AMY_r ACTCAAGGTAAGTAACAGCCCACGG, AMY_f CTACACGTGGCTTGGTCACTTCATG; "spike"-DNA: mhvM2a GGTGATGGGACAGACGGGAACTAC, mhvB1 CTGGTGCCACTCAGAATGTTCAGTG; ark222amp5 GTGCGCGGAACCCCTATTTG, ark223amp3 GGTCTGACGCTCAGTGGAACG; viral DNA, EBV: repeat_r AGCAGCGCAGCCAACCATAGAC, repeat_f CCCAAAGCGGGTGCAGTAACAG; 5′-OriP_r ATGTCTGCCATAGTTTCGAGTGGTG, 5′-OriP_f TCAGTCACGGGACATTAACCTCAAC; OriP_r TGATTGTAACCCCGCTAACCACTG, OriP_f CGTGAATATGAGGACCAACAACCC; 3′-OriP_r CACTGCAGAGTGACCACTAACCTTCG, 3′-OriP_f AAATACGTCCTACCCAGGAACCCG; BVRF1_r TGGTCACGCATGATGCCGTC, BVRF1_f TCCGGGCACGTCTTGATAGACC; LMP1_r TGATGGAGGCCCTCCACAATTG, LMP1_f CATAGTAGCTTAGCTGAACTGGGCCG; BRLF1_r TGATGAAGAAACCAGTCAGGCCG, BRLF1_f TTCATTAAGTTCGGGGGTCAGGG. HVS: H-DNA_r TGGGGACCTGAGGGTTTTGG, H-DNA_f TGACGGCTGCAAACTCTGGC; Orf1_r AGAGAAATGTGTCAGACAAGAAGTGGG, Orf1_f GTGTTGCTCTTGTTAGCTGTTTCTTGTG; Orf25_r AAGTGCTACAGGAACAAGAGGTCCTATG, Orf25_f CATGCACATGTAATCTATAGGTGTCTGCTC; Orf50_r GTCTATGGGTGGAAACAATTGCATG, Orf50_f TAAGCCTAGTGTCCGACTTACATTTTGC; Orf73_r GGTTTAACATATGTTTTGCGGTTGC, Orf73_f GTGCTACTCACATTGAAAATCGAAACTTC.

Quantitative SYBR-Green PCR with sorted cells

Six cell cycle fractions were sorted into separate vials (100,000 cells each), lysed and equal amounts of MHV68 "spike"-DNA were added as an internal standard to control for uneven DNA isolation and precipitation. The DNA was then isolated by phenol–chloroform extraction and precipitated with ethanol; sorted Raji B cells were analyzed directly, with or without BrdU immunoprecipitation (Fig. 3). Quantitative SYBR-Green PCR was performed in 1× PCR buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl, 1.5 mM $MgCl_2$), 0.5 mM dNTP, 2.5 U Taq-Polymerase, 0.3 µM Rox passive reference dye, 0.6 µl of SYBR-Green-I (10× diluted from 10,000× stock solution, Invitrogen), 10 pM of each primer. As a control, cellular primers specific for genes with known replication timing (beta-globin gene, early; alpha amylase gene, late) were used. The detection of viral DNA was done with primers within the OriP and the BVRF1 gene. The Ct value for both viral and cellular DNA obtained from qPCR of the G1 fraction was set to one "relative diploid genome copy". This was done to facilitate the visualization of the target DNA copy number increase throughout the cell cycle fractions. The quantitative PCR was done once in duplicates (data not shown) and once in triplicates with comparable results.

Fig. 5. Relative abundance of locus-specific signals in each cell cycle fraction. Signals obtained by quantitative SYBR-Green PCR of BrdU-immunoprecipitated, newly replicated DNA (histogram plot, percentage of total signal). (A) HVS. The upper two rows show the results for the cellular controls with known replication timing. The results for the HVS episomal DNA are divided into genes active during latency and genes only active during lytic replication but silent during latency and the repetitive H-DNA region. The results for all loci demonstrate an early replication timing of the episomal DNA of HVS. The genes in heterochromatin (orf50 and orf25) exhibit a slightly delayed replication compared to loci in the euchromatic region (orf1, orf73, and H-DNA) of the episome. (B) EBV. The upper two rows show the results for the cellular controls with known replication timing. The results for the EBV episomal DNA are divided into genes active during latency (LMP1) and the loci in the repeat region plus inside and at the 3′/5′ end of the OriP. All these analyzed loci demonstrate an early replication timing of the episomal DNA of EBV.

Quantitative SYBR-Green PCR after BrdU immunoprecipitation

Cells were sorted and "spike"-DNA added as above; following BrdU immunoprecipitation, the relative abundance of isolated DNA in each fraction was determined by locus-specific quantitative SYBR-Green PCR. For each primer pair, at least duplicates, in most experiments quadruplicate PCR reactions were analyzed. The resulting Ct threshold values of each fraction were exported into an Excel spreadsheet. Values of quantities of "spike"-DNA in the respective fractions were used to normalize the BrdU-precipitated DNA recovery. The relative abundance of locus-specific DNA in each fraction was calculated by inserting the average values of the duplicates/quadruplicates into the following equation: $(2^{-Ct} / \Sigma 2^{-2Ct})$. The data were normalized to the total signal of all fractions as in the work by Azuara et al. (2003).

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