Epstein–Barr virus origin of lytic replication mediates association of replicating episomes with promyelocytic leukaemia protein nuclear bodies and replication compartments

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Epstein–Barr virus (EBV) establishes a latent persistence from which it can be reactivated to undergo lytic replication. Late lytic-cycle gene expression is linked to lytic DNA replication, as it is sensitive to the same inhibitors that block lytic replication, and it has recently been shown that the viral origin of lytic replication (ori lyt) is required in cis for late-gene expression. During the lytic cycle, the viral genome forms replication compartments, which are usually adjacent to promyelocytic leukaemia protein (PML) nuclear bodies. A tetracycline repressor DNA-binding domain–enhanced green fluorescent protein fusion was used to visualize replicating plasmids carrying a tetracycline operator sequence array. ori lyt mediated the production of plasmid replication compartments that were associated with PML nuclear bodies. Plasmids carrying ori lyt and EBV itself were visualized in the same cells and replicated in similar regions of the nucleus, further supporting the validity of the plasmids for studying late-gene regulation.

Like all herpesviruses, the life cycle of Epstein–Barr virus (EBV) is biphasic. After EBV establishes latency in the human host, it can be reactivated to enter the lytic cycle. During latency, the EBV genome persists as a closed, circular episome. Similar to the host chromosomal DNA, it is packaged in nucleosomal arrays with cellular histones (Dyson & Farrell, 1985), replicates once during the S phase of the cell cycle (Adams, 1987) and is divided between the daughter cells during mitosis (Yates et al., 1984).

In contrast to latent replication, multiple rounds of replication are initiated within the viral origin of lytic replication (ori lyt) during the lytic cycle (Hammerschmidt & Sugden, 1988). ori lyt-mediated replication involves two different phases. At first, the viral episome is amplified to yield monomeric plasmid progeny (theta mode). In the second phase, the viral genome is amplified 100- to 1000-fold through a rolling-circle mechanism, leading to large, head-to-tail concatemeric molecules that are subsequently cleaved into single EBV genomes (Pfüllér & Hammerschmidt, 1996). Late-gene expression is linked to lytic DNA replication, as it is sensitive to the same inhibitors (phosphonoacetic acid and acyclovir) that block lytic replication (Summers & Klein, 1976).

We recently showed that the expression of late-lytic genes depends on the presence of ori lyt in cis on stably transfected luciferase reporter plasmids (Amon et al., 2004). Efficiency of lytic replication, late-gene expression and sensitivity to inhibitors were functions of the size of ori lyt used. With the so-called big ori lyt (bol), which comprised the BamHI H fragment of EBV, we properly reconstituted lytic DNA replication and late-gene expression on small plasmids. Our results contrasted with those of Serio et al. (1997), who found no requirement for the ori lyt sequence in cis for late-gene expression in a transient assay, implicating a trans-acting factor. Because of this difference, we have further characterized the importance of the EBV ori lyt, analysing the nuclear location of small plasmids containing ori lyt during the lytic cycle.

Lytic DNA replication occurs at discrete sites in the nucleus, called replication compartments (Daikoku et al., 2005). Promyelocytic leukaemia protein nuclear bodies (PML-NBs), speckled subnuclear structures with reported functions in tumour suppression, apoptosis and interferon-regulated antiviral defence (Ahn & Hayward, 2000; Guo et al., 2000; Li et al., 2000), are found co-localized with the replication compartments. EBV has been reported to only be associated with PML-NBs during the lytic cycle as replication compartments develop, and not during latency (Bell et al., 2000). During replication, the PML-NBs become disrupted. Although the association of EBV with PML-NBs during lytic replication is established, which viral sequences are required for this process was so far unknown. We show here that ori lyt is required for the appearance of replicating...
episomes that are associated with PML-NBs. Furthermore, plasmids carrying ori lyt frequently occupy the same replication compartments as EBV in a pattern that suggests that replication compartments may derive from one or a few episomes.

The plasmid pMOVE-bol, carrying ori lyt (Amon et al., 2004), was visualized under the confocal microscope through multiple binding of an enhanced green fluorescent protein (EGFP)-fusion protein (Fig. 1a), using a strategy similar to that of Sourvinos & Everett (2002). The plasmid contains multiple arrays (100–150 copies) of the tetracycline operator sequence (TetO), which is recognized by the tetracycline repressor DNA-binding domain (TetR). TetR was expressed as a fusion with EGFP. Expression of the protein was verified under the fluorescence microscope (Fig. 1b, left). As a control, cells expressing an EGFP–nuclear localization signal (nls) fusion protein lacking the TetR domain were also generated (Fig. 1b, middle). The replicating plasmids binding EGFP–nls–TetR could be detected through confocal imaging. For immunofluorescence, cells were transferred to Cytospin microscope slides and fixed in a 1:1 solution of ice-cold methanol and acetone for 30 min at $-20^\circ$C, which reduced the background level of unbound EGFP.

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![Diagram](image)

**Fig. 1.** (a) Plasmid pMOVE is a bacterial artificial chromosome (BAC) carrying the big ori lyt and the multiple Tet operator-binding array (TetO). pMOVE also contains oriP for plasmid maintenance. EGPF–nls–TetR is expressed under the control of the cytomegalovirus immediate-early promoter from a separate plasmid. (b) Fluorescence microscopy of cells transfected with the EGFP–nls–TetR expression plasmid (left), the EGFP–nls expression plasmid (middle) and untransfected cells (right). (c) Visualization of replicating plasmids. Confocal image of Akata 2003 cells showing the formation of plasmid replication compartments appearing as green dots (arrowed) 48 h post-induction. The replication compartments only appear in induced cells carrying pMOVE-bol (big ori lyt) and Puro:EGFP–nls–TetR (left). Replication compartments were not visible in Akata 2003/pMOVE-bol cells expressing the EGFP protein without the TetR DNA-binding domain (right). (d) Immunofluorescence of cells stained for replicating plasmids (EGFP, green) and the single-stranded DNA-binding protein BALF2 (red) 10 h post-induction. BALF2 was stained with antibody OT13B (Zeng et al., 1997).
By using this system, it was possible to visualize the plasmid replication compartments, where the concatemeric TetO plasmids are concentrated in certain areas of the nucleus during the EBV lytic cycle (Fig. 1c). The left image shows several cells containing the TetO plasmids with the ori lyt (pMOVE-bol) and the EGFP–nls–TetR expression plasmid. To disrupt latency, cells were treated with anti-IgG for 48 h. In the cells that have entered the lytic cycle, the plasmid pMOVE-bol underwent lytic DNA replication and, through binding of the EGFP-fusion protein to the replicating plasmids, this was observed as green dots (see arrows). Each dot represents one replication compartment. The occurrence of these green dots is not a result of an imbalanced distribution of the EGFP-fusion protein in the cells, but depends on the binding of EGFP–nls–TetR to the plasmids carrying the TetO-binding sites. In cells expressing the EGFP–nls protein without TetR, which cannot bind to the TetO plasmids, no green dots were detected (Fig. 1c, right image). The individual plasmid episomes were not detectable during latency with this system against the background level of EGFP–nls–TetR.

The single-stranded DNA-binding protein BALF2, part of the replication complex, co-localized with the replicating plasmids, confirming the observed green areas to be replication compartments (Fig. 1d). Complete co-localization was not expected, as EBV genomes are also replicating in the same cells.

We previously demonstrated (Amon et al., 2004) that small plasmids containing bol replicate in parallel with the endogenous EBV genome. bol supported late-gene expression and mediated sensitivity to inhibitors of lytic DNA replication. As these plasmids reflected the behaviour of the virus closely, it seems likely that the presence of ori lyt plays a key role in these events. We therefore tested whether, when plasmids carrying bol form their replication sites, PML-NBs could be found in the vicinity, as was observed for EBV (Bell et al., 2000). A rabbit polyclonal antibody against human PML (Santa Cruz) was used as a primary antibody and a goat anti-rabbit IgG–tetramethylrhodamine isothiocyanate conjugate served as the fluorescent secondary antibody. To detect cells that have entered the viral lytic cycle, the EBV immediate-early protein BZLF1 was also stained (BZ-1 mouse mAb and goat anti-mouse IgG–Cy5 conjugate). Akata 2003 cells transfected with the EGFP–nls–TetR expression vector and the plasmid pMOVE-bol were induced with anti-IgG and samples were taken after 6, 12 and 24 h. Fig. 2(a) shows the cells after treatment with anti-IgG for 6 h. Induced cells that have entered the lytic cycle (indicated) express BZLF1. At this early stage of the lytic cycle, the PML-NBs (red) are still intact. Plasmid replication has not yet started, as judged by the absence of green dots (EGFP–nls–TetR) indicating replication compartments.

Twelve hours after induction of the lytic cycle (Fig. 2b), replication sites have started to form (green). In many cases, these sites were in the vicinity of PML-NBs (red), indicating an association between plasmid replication factories and PML-NBs (see arrows). However, not all sites of plasmid

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**Fig. 2.** (a) Replication compartments (green) are not yet detectable 6 h post-induction. The PML-NBs (red) are still intact. Cells that have entered the lytic cycle (indicated) express BZLF1. (b) Replication compartments (green) have formed 12 h post-induction. Cells that have entered the lytic cycle (indicated) express BZLF1 (blue). PML-NBs frequently form in association with PML-NBs (red) (arrows). (c) Disruption of PML-NBs 12 h after induction. Some cells were found to contain enlarged red spots (arrows). (d) PML-NBs are not detected after 24 h in those cells that have entered the lytic cycle. Plasmid replication compartments (EGFP) are shown as green spots. Cells were stained for PML (red) and BZLF1 (blue).
replication shown in the images have a PML body next to them. In many cases, the PML-NBs were not in the same confocal plane, as three-dimensional projections revealed. In other cases, the PML-NBs might already be disrupted. Some of the cells in the lytic cycle were found to have abnormally large PML-NBs (Fig. 2c). This was never observed in latent cells and thus seems to be connected with the EBV lytic cycle. Interestingly, these enlarged PML-NBs were always close to a plasmid replication compartment. The enlarged red areas could therefore represent disrupting PML-NBs that are dispersing their PML protein. After 24 h treatment with anti-IgG, all PML-NBs have been disrupted in those cells that have entered the EBV lytic cycle (Fig. 2d).

The results of the immunofluorescence experiments indicate an association between plasmid replication sites and PML-NBs, similar to that of the viral genome. The plasmids also contain the EBV oriP sequence, so we cannot exclude the possibility that this makes a contribution, but it is likely that ori lyt is sufficient for the replication and association with PML-NBs.

The observed association of replicating ori lyt plasmids with PML-NBs still leaves the question of whether the plasmids actually replicate at the same sites in the nucleus as the EBV genome. The system described here allows observation of two independently replicating types of DNA within the same cell. For this purpose, the ori lyt plasmids and the viral episomes were stained with two different colours by using fluorescence in situ hybridization (Fig. 3a). Confocal imaging revealed the formation of plasmid (green) and virus (red) replication compartments 12 h after induction of the lytic cycle (Fig. 3b). Only certain areas of the cell nuclei contained lytically replicating DNA. With a few exceptions, these replication areas contain both replicating virus and plasmid DNA. Replicating plasmids appear more focused, because each green dot represents a large, concatemeric molecule of several hundred plasmid copies that cannot be cleaved due to the lack of terminal repeats. In contrast, the viral DNA (red) is spread over a larger area, presumably because the single virus amplicons are free to diffuse in the nucleus or are transported actively to the sites of virus assembly. This co-localization of virus and plasmid DNA supports the conclusion that the ori lyt sequence is sufficient to mediate the association of small plasmids with the virus replication compartments. The frequent co-localization of plasmid and viral genomes in replication compartments, with a few containing either plasmid or viral DNA uniquely, suggests that each replication compartment derives from one or a few initial plasmid or viral episomes.

After finding that the ori lyt sequence is required in cis for late-gene expression (Amon et al., 2004), a process linked to lytic DNA replication through inhibitors such as phosphonoacetic acid and acyclovir, we have now shown that ori lyt is also required for the production of virus replication compartments associated with PML-NBs. Our results do not exclude the possible contribution of a trans-acting factor to EBV late-gene expression (Serio et al., 1997), but in herpes simplex virus 1 (HSV-1), the viral origin of replication OriS is sufficient for the association of plasmids with PML-NBs (Tang et al., 2003). Similarly, in Simian virus 40, the minimum sequence required for the association with PML-NBs contained the viral core origin of replication, although the origin alone was not sufficient (Tang et al., 2000).

Association of the viral episome with PML-NBs during reactivation might contribute to the link between lytic DNA

Fig. 3. (a) Fluorescence in situ hybridization. Viral DNA was hybridized with a digoxigenin (DIG)-labelled probe against part of the BamHI C fragment and detected with an anti-DIG rhodamine-conjugated antibody. The ori lyt plasmid pMOVE-bol was detected with a biotin-labelled probe against the BAC backbone and avidin–fluorescein. Cells were fixed in a 1 : 1 solution of ice-cold methanol and acetone for 30 min at −20 °C and denatured in 70% formamide in 2 × SSC for 15 min at 80 °C. (b) EBV (red) and the ori lyt plasmid pMOVE-bol (green) form their replication compartments in the same areas of the nucleus (12 h post-induction).
replication and late-gene expression. All three processes (association with PML-NBs, lytic replication and late-gene expression) depend on the presence of ori lyt in cis. Upon reactivation, the viral episome was located in an ori lyt-dependent manner adjacent to PML-NBs. These are not only the sites for lytic DNA replication, but might also be sites of active transcription (Everett, 2001), which could promote late-gene expression.

The plasmid system allowed us to study two independently replicating units during the lytic cycle within the same cell. Our fixed-time data cannot distinguish whether replicating EBV plasmids move to become adjacent to PML-NBs, as previously suggested for EBV reactivation (Bell et al., 2000), or whether the PML-NBs form adjacent to replication foci, as was concluded in recent studies of HSV-1 infection (Everett et al., 2004; Everett & Murray, 2005). However, the similar behaviour of the small plasmids containing ori lyt and the EBV genome in nuclear location is consistent with our earlier argument that the plasmid expression system that we used to study late-gene regulation (Amon et al., 2004) has characteristics very similar to those of the complete viral genome.

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References


