Long-term replication of Epstein-Barr virus-derived episomal vectors in the rodent cells

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Abstract Plasmids containing the origin of replication, oriP, of the Epstein-Barr virus (EBV) and EBV nuclear antigen-1 genes replicate extrachromosomally in primate cells. However, these plasmids have been believed not to replicate in rodent cells. We demonstrate here that these plasmids can replicate in some types of rodent cells over a long period. This result should offer not only the new insight into the mechanisms of species-specific replication of EBV, but also the possibility that an EBV-based vector can be used for gene transfer experiments in non-primate cells and an animal experiment regarding human gene therapy.

Key words: Epstein-Barr virus; Episomal vector; Gene therapy; Rodent cell

1. Introduction

Episomal vectors which replicate extrachromosomally in cells over a long period are useful tools for studying the expression of cloned genes and gene therapy [1]. Epstein-Barr virus (EBV)-derived plasmid is one of the promising candidates for use in these types of studies [2–4]. The viral elements required for episomal replication and nuclear retention are the *cis*-acting replication origin (oriP) of the EBV gene and the EBV nuclear antigen-1 (EBNA-1), which interacts with the oriP region [2–4]. Plasmids containing the oriP and EBNA-1 sequences are maintained as low-copy number DNA episomes in the cell nucleus and replicate once per cell cycle in primate cells [5].

OriP is composed of two clusters of the EBNA-1 binding region: a family of repeat and a dyad symmetry sequence [3,6]. Both elements have multiple binding sites for EBNA-1, and are essential for replication and nuclear retention of the plasmid containing oriP [7]. The dyad symmetry sequence is important in the initiation of DNA replication [8,9], while the family of repeat acts as an EBNA-1-dependent enhancer and is responsible for DNA replication and for the stable segregation of the viral episome [3,10,11]. Host cellular factors assist the replication and nuclear retention of the plasmid containing both the oriP and the EBNA-1 sequences [12].

Several previous reports have demonstrated that the long-term replication of EBV-derived plasmid occurs in primate cells but not in rodent cells [2,13]. In rodent cells, EBV-derived plasmid seems to have only the nuclear retention function and the enhancer activity of transgene expression induced by EBNA-1 [14–16]. Specific factors derived from primate cells have been believed to be required for long-term replication and maintenance of EBV-derived plasmid.

In this study, we demonstrate that plasmids containing the oriP and EBNA-1 sequences can replicate in some kinds of rodent cells. Long-term replication of EBV-derived plasmid in their cell lines was shown by colony formation assay, sustained transgene expression and Southern blot analysis using the difference of methylation of input DNA and replicated DNA in the mammalian cells.

2. Materials and methods

2.1. Plasmid

An EBV-derived plasmid, pREP10-L1, contains the oriP and the EBNA-1 gene which is derived from pREP10 (Invitrogen, Carlsbad, CA, USA), the hygromycin-resistant gene, and the luciferase gene which is driven by the Rous sarcoma virus long terminal repeat (RSV-LTR) promoter (Fig. 1A). The EBNA-1 gene in pREP10-L1 codes the truncated protein (the deletion of GlyAla repeat domain).

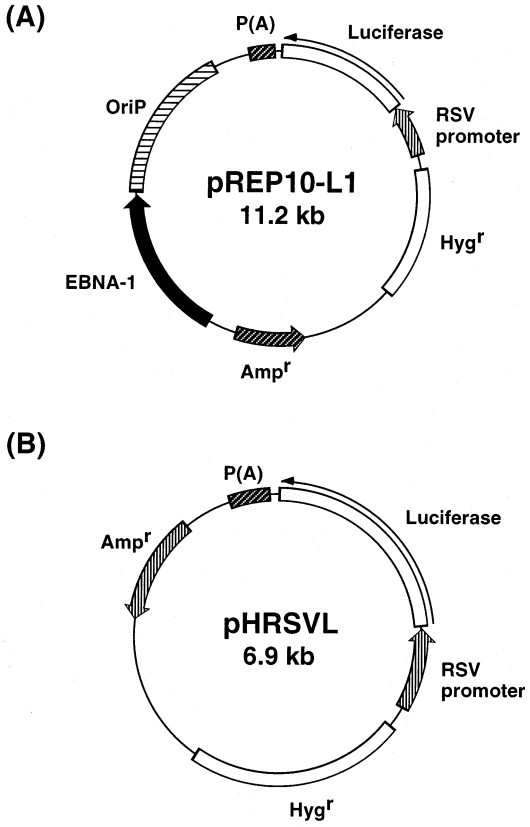

Fig. 1. The plasmid structure. (A) pREP10-L1, (B) pHRSVL.

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pHRSVL contains the luciferase gene driven by the RSV-LTR promoter and hygromycin-resistant gene, but does not contain oriP or the EBNA-1 gene (Fig. 1B).

2.2. Cells

HeLa cells (from human epitheloid carcinoma of the cervix, obtained from Dr. M.A. Kay, Stanford University, USA), RL-34 cells (from rat liver epithelial cells, Human Science Research Resources Bank (HSRRB), Japan, JCRB0247) and L6 cells (from rat skeletal muscle myoblast; HSRRB, JCRB9081) were cultured with Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). L cells (Lcl1D, from mouse skin fibroblast; HSRRB, JCRB0722) and NIH3T3 cells (from mouse embryo fibroblast; HSRRB, JCRB0615) were cultured with minimum essential medium (MEM) supplemented with 10% FCS. BHK-21 cells (from hamster kidney; HSRRB, JCRB9020) were cultured with MEM supplemented with 10% FCS and non-essential amino acids. Chinese hamster ovary (CHO) cells (obtained from Dr. T. Mayumi, Osaka University, Japan) were cultured with MEM alpha medium supplemented with 10% FCS. C6 cells (from rat glioma; HSRRB, JCRB9096) were cultured with Ham's F10 medium supplemented with 10% FCS. In the colony formation assay, the cells were selected with the following concentrations of hygromycin B (μg/ml): BHK-21 and NIH3T3, 60; HeLa, 200; C6 and RL-34, 350; CHO and L6, 500; L, 600.

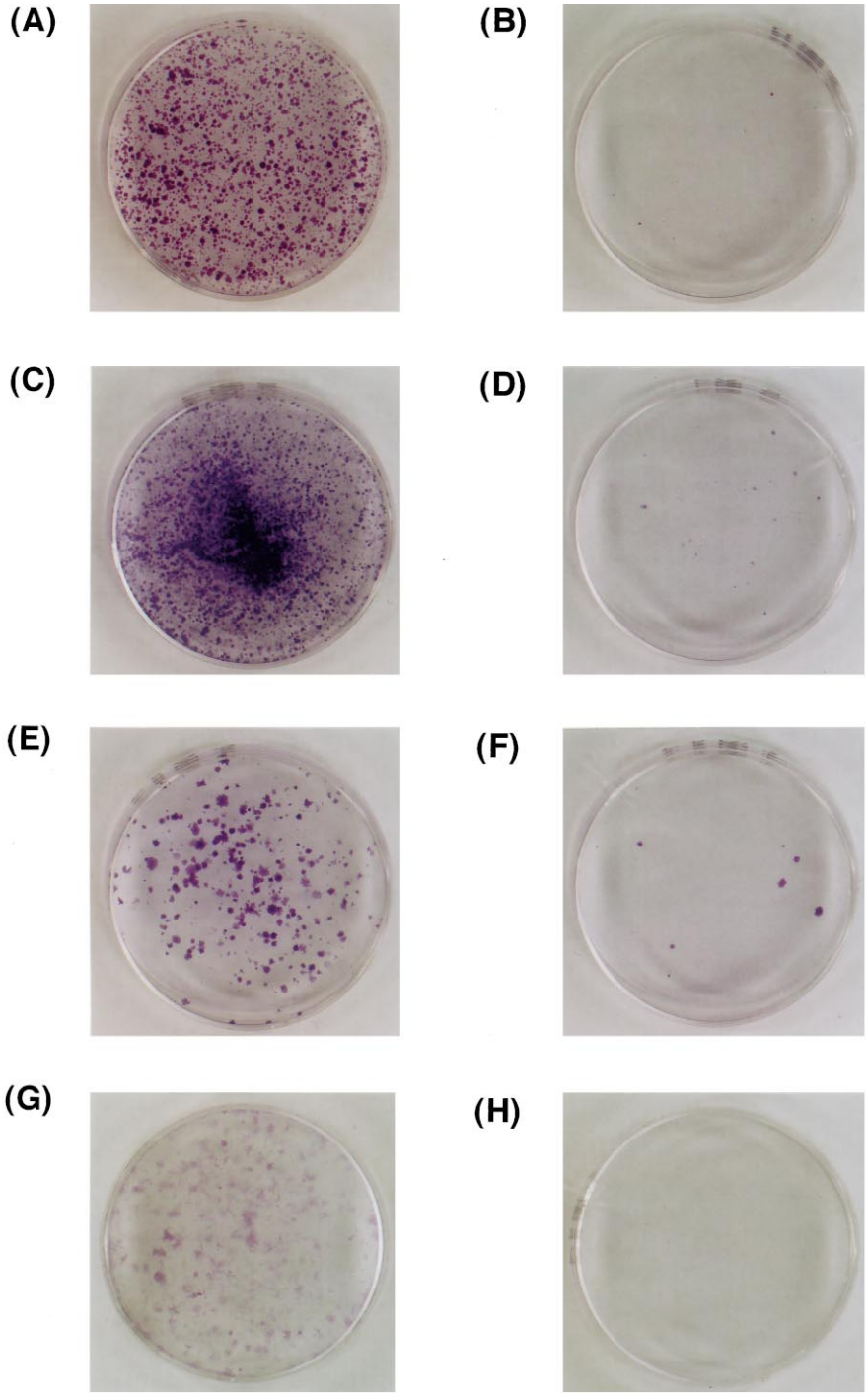

Fig. 2. Efficiency of colony formation of various cell lines transfected by pREP10-L1 or pHRSVL. HeLa (A, B), L (C, D), L6 (E, F) and C6 (G, H) cells were transfected by pREP10-L1 (A, C, E, G) or pHRSVL (B, D, F, H) using Superfect. On the following day, the cells were passaged and cultured with medium containing hygromycin B. After 12–14 days of culture, the cell colonies were stained with crystal-violet.

2.3. Colony formation assay

The cells (1×10^5 cells) were seeded into 12-well dishes. On the following day, pREP10-L1 or pHRSVL (1.5 μg) were transfected with Superfect (Qiagen) for 2 h according to the manufacturer's instructions. The DNA amount was corrected using the plasmid pUC18 to transfect equal molars of pREP10-L1 and pHRSVL. 24 h later, the cells were detached and suspended with fresh medium (final volume, 10.0 ml), and 5.0 ml of the cell suspension was seeded into a 60 mm dish. The following day, the medium was changed to that containing hygromycin B. After 12–14 days in culture, the cell colonies were stained with crystal-violet.

2.4. Luciferase assay

pREP10-L1 or pHRSVL (1.0 μg) were transfected into the cells (5×10^4 cells) according to the same protocol as that used in the colony formation assay. After 2, 4, 6 and 8 days in culture with normal medium without hygromycin B, luciferase activity in the cells was measured using a luciferase assay system (PicaGene LT2.0, Toyo Inki Co. Ltd., Tokyo, Japan). We repeated all transfection experiments, including the colony formation assay, and obtained similar results.

2.5. Southern blot analysis

Hygromycin B-resistant cell colonies (HeLa, L6, C6 and L) transfected by pREP10-L1 were grown as pools. After 2 months in culture, low molecular weight DNA was prepared as described by Hirt [18]. Hirt supernatant DNA was digested by *Xba*I (single cutter), *Xba*I/*Mbo*I or *Xba*I/*Dpn*I, electrophoresed on 0.7% agarose gels, and transferred to a Hybond nylon filter (Amersham, Buckinghamshire, UK). The blots were hybridized using [α-^{32}P]dCTP-labelled DNA probes (*Bam*HI/*Not*I fragment (hygromycin gene) of pREP10-L1).

3. Results

3.1. Colony formation of hygromycin-resistant cells transfected by EBV-based vector

When the colony formation assay in the cell lines (most of human origin) transfected by EBV-derived plasmids (i.e. pREP10-L1) (Fig. 1) was performed, we found that mouse L cells transfected by EBV-derived plasmid had a much greater number of hygromycin-resistant colonies than did those transfected by pHRSVL, which contained the luciferase expression cassette and the hygromycin B-resistant gene, but not oriP or EBNA-1 gene. This finding led us to speculate that some kinds of rodent cells can support the long-term replication of EBV-based plasmid, although it has not been believed that EBV-based plasmid replicates in rodent cells [2,13]. Therefore, we examined the long-term replication of EBV-based plasmid in various kinds of rodent cells by using colony formation assay.

Mouse (L, NIH3T3), rat (L6, C6, RL-34), hamster (CHO, BHK-21) and human (HeLa)-derived cells were transfected by pREP10-L1 or pHRSVL, and the number of hygromycin-resistant colonies was compared (Fig. 2). HeLa, L and L6 cells transfected by pREP10-L1 had about 500, 1000 and 30 times more colonies than those transfected by pHRSVL, respectively. C6 cells transfected by pREP10-L1 showed a number of colonies, while the cells transfected by pHRSVL showed none in the condition of this study. In contrast, in NIH3T3, RL-34, CHO and BHK-21 cells, the colony numbers were similar between pREP10-L1 and pHRSVL (data not shown). The hygromycin B-resistant colony transfected by pHRSVL should reflect the integration of the plasmid (hygromycin-resistant gene) into the genomic chromosomes of the cells. Thus, the larger number of resistant colonies in pREP10-L1-transfected cells might account for the long-term episomal replication of the plasmid outside the chromosome. pREP4-L1, which has the EBNA-1 gene containing the GlyAla repeat domain derived from pREP4 (Invitrogen), also showed similar results with pREP10-L1, which contains the truncated EBNA-1 gene (data not shown).

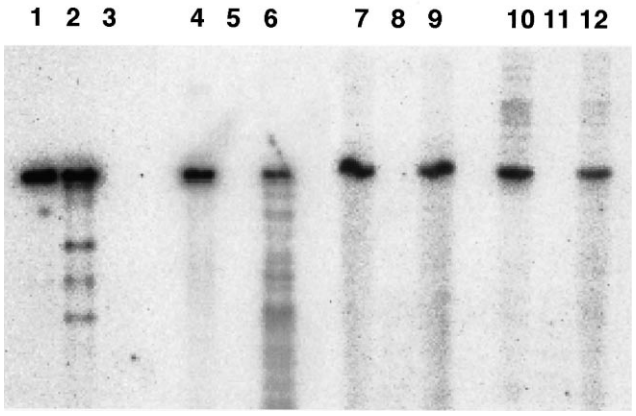

Fig. 3. Replication analysis of pREP10-L1 in various cell lines. Hirt supernatant DNAs were prepared from HeLa (lanes 4–6), L6 (lanes 7–9) and C6 (lanes 10–12) cells transfected by pREP10-L1. Each cell was cultured for 2 months. DNA was digested by *Xba*I (single cutter) (lanes 1, 4, 7, 10), *Xba*I/*Mbo*I (lanes 2, 5, 8, 11) or *Xba*I/*Dpn*I (lanes 3, 6, 9, 12). As a control, plasmid pREP10-L1, which was prepared with *E. coli*, was also analyzed (lanes 1–3). Hygromycin gene was used as a DNA probe.

3.2. Southern blot analysis of episomal replication of EBV-based vector

To examine the episomal replication of pREP10-L1 in HeLa, L, L6 and C6 cells, low molecular weight DNAs were isolated by Hirt's method [17] from the hygromycin B-resistant cells. Hirt DNAs were then digested by *Xba*I (one cutter), *Xba*I/*Mbo*I or *Xba*I/*Dpn*I, electrophoresed and subjected to Southern blotting. *Mbo*I cut the DNA which had lost the bacterial methylation, while *Dpn*I cut the methylated DNA. Therefore, input DNA, which was prepared from *Escherichia coli*, is susceptible to *Dpn*I (but not to *Mbo*I), while the DNA which had been replicated in the mammalian cells is susceptible to *Mbo*I (but not to *Dpn*I).

As seen in Fig. 3, Hirt DNAs from HeLa, L6 and C6 cells were digested by *Mbo*I, but not by *Dpn*I. In contrast, pREP10-L1 prepared with *E. coli* was digested by *Dpn*I, but not by *Mbo*I, which showed the opposite pattern as that of Hirt DNA. These results suggest that pREP10-L1 did replicate in the rodent cells (L6 and C6 cells) as well as in the primate cells (HeLa cells) over a long period. In spite of considerable efforts, we could not detect any signals in Southern blotting using both Hirt DNA and whole genomic DNA recovered from pREP10-L1-transfected L cells, both of which were resistant to hygromycin B and expressed luciferase. However, taking into consideration the Southern blotting of pREP10-L1-transfected L6 and C6 cells and the colony formation assay shown in Fig. 2, L cells might also support the long-term replication of pREP10-L1.

3.3. Prolonged and enhanced luciferase expression by EBV-based vector

Next, we examined whether pREP10-L1 causes more prolonged and enhanced luciferase expression in rodent cells than does pHRSVL due to the retention [18,19], replication [2,4]

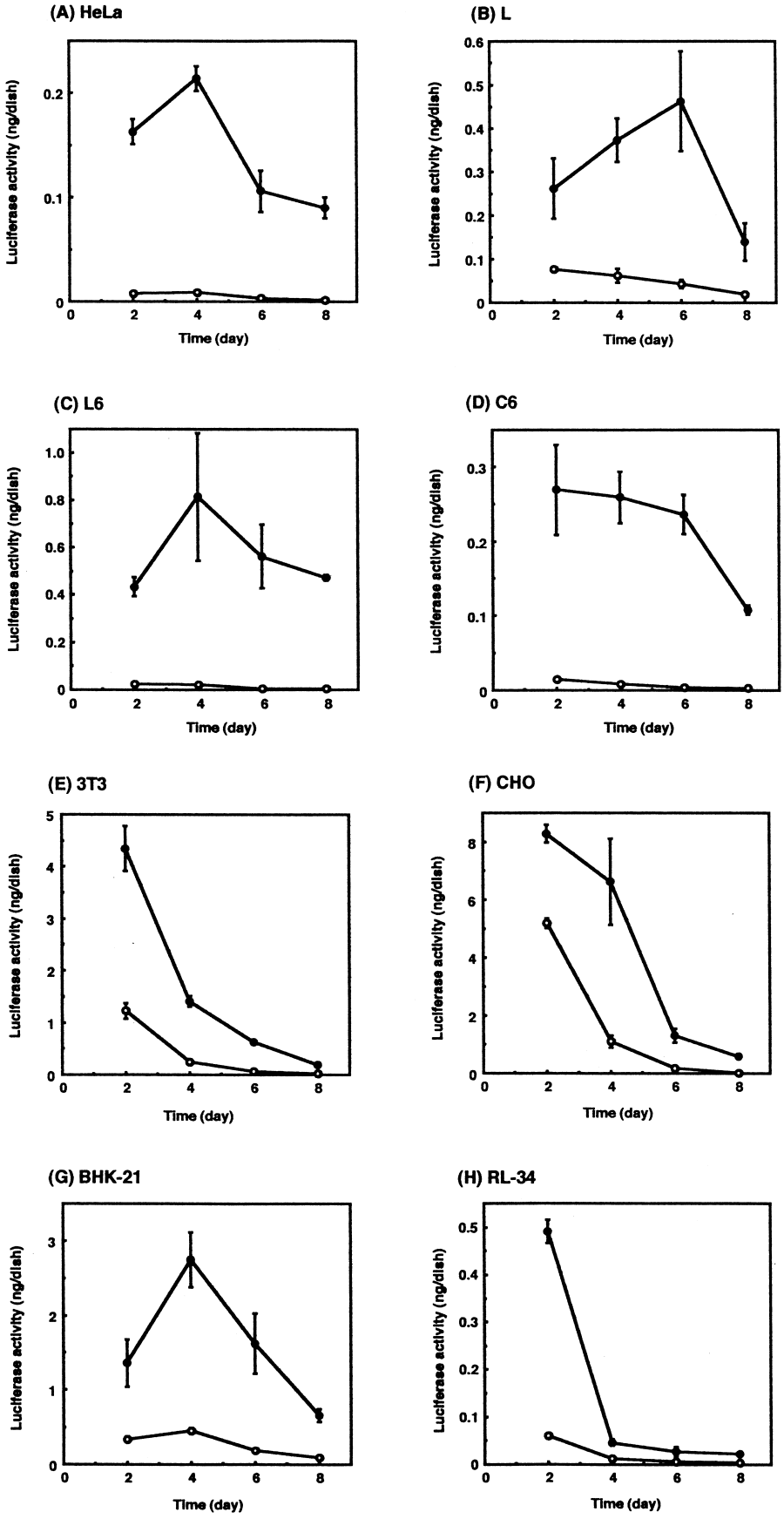
(A) HeLa
(B) L
(C) L6
(D) C6
(E) 3T3
(F) CHO
(G) BHK-21
(H) RL-34
Luciferase activity (ng/dish)
Time (day)

Fig. 4. Duration of luciferase expression in various cell lines transfected by pREP10-L1 or pHRSVL. HeLa (A), L (B), L6 (C), C6 (D), NIH3T3 (E), CHO (F), BHK-21 (G) and RL-34 (H) cells were transfected by pREP10-L1 (closed circle) or pHRSVL (open circle) with Superfect. After 2, 4, 6 and 8 days, luciferase expression in the cells was measured by luminescent assay. All data represent the mean ± S.D. of three experiments.
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and enhancing activity [10,11] induced by the EBV-derived fragment (Fig. 4). All cell lines transfected by pREP10-L1 showed higher luciferase expression than those transfected by pHRSVL. In HeLa, L, L6 and C6 cells, which supported the long-term replication of pREP10-L1, prolonged luciferase expression was also observed by the transfection of pREP10-L1 (Fig. 4A–D). In contrast, in 3T3, CHO, BHK-21 and RL-34 cells, longer duration of luciferase expression was not observed (Fig. 4E–H). These results suggested that the EBV-derived fragment up-regulated transcription of the RSV promoter-driven luciferase gene in all cell lines examined, and that in some cell lines, prolonged luciferase expression was also observed due to the replication activity of pREP10-L1. In this study, we saw no evidence of retention activity of pREP10-L1 in the cell lines which did not support the replication of pREP10-L1.

4. Discussion

In this paper, we demonstrated that the plasmid containing the oriP and EBNA-1 genes did replicate efficiently in some kinds of rodent cells as well as in human (primate) cells. Yates et al. developed an EBV-derived episomal vector and showed that the vector replicates in primate cells, but not in rodent cells [2]. Since then, rodent cells have been believed to be non-permissive for the replication of the EBV-derived vector [13]. Therefore, host cellular factor(s) derived from primate cells have been believed to cooperate with EBNA-1 to mediate plasmid replication. However, only limited kinds of rodent cells have been analyzed in terms of plasmid replication so far. We analyzed the long-term replication of EBV-derived plasmid in seven kinds of rodent cells. Among them, three kinds of cells (L, L6, C6) supported the long-term replication of EBV-derived plasmid efficiently (Figs. 2 and 3).

Only two reports have described the successful use of EBV-derived vectors in rodent cells, rat C6 glioma cells [15] and rat pheochromocytoma PC12 cells [20], although these studies did not analyze the use of these vectors in great depth. In contrast, one report described that even human-derived cells (HT-1376 bladder carcinoma cells) did not support the replication of EBV-derived plasmid [21]. These results, combined with our present data, suggest that other factor(s) might play more important roles in the long-term replication of EBV-derived plasmid in cells than might species-specific factor(s).

EBV-derived plasmid, pREP10-L1, also showed higher luciferase expression in both human and rodent cells than did the plasmid without the EBV fragment, pHRSVL (Fig. 4). This is consistent with previous reports [14–16]. The EBNA-1–oriP system has several functions other than plasmid replication, such as the nuclear retention of the plasmid containing oriP [18,19], and the transactivation of EBV transcriptional enhancer by EBNA-1 [10,11]. These functions would enhance the induction of luciferase expression by pREP10-L1, although the retention activity of pREP10-L1 was not confirmed in 3T3, CHO, BHK-21 or RL-34 cells.

Several research groups developed EBV-derived vectors which contain large fragments of human genomic DNA as well as the EBNA-1 gene and the family of repeat sequence [1,13,22–26]. Such vectors were reported to replicate in rodent cells [13,22]. Calos and colleagues showed that hamster BHK cells support the long-term replication of modified EBV-based vectors carrying large fragments of human DNA, but not that of the wild-type EBV-based vector [13]. Although we demonstrated that EBV-based vectors could replicate in some kinds of rodent cells, the modification of the vector described by Calos et al. would broaden the tropism of the EBV-based episomal vector.

One of the problems of EBV-based vectors in regard to their application in gene therapy is that there is no small animal model which supports the replication of the vector. Recently, prolonged and enhanced transgene expression in mice liver and muscle transduced by the EBV-based vectors has been reported [16,27]. Plasmid replication might have played a role in these phenomena, although the authors discussed that sustained gene expression was only due to nuclear retention and not the replication of the vector.

In summary, ours is the first report to clearly demonstrate that EBV-derived plasmid replicates in rodent cells as well as in primate cells. Taken together with the long-term replication and enhanced transgene expression induced by EBV-based vectors in the rodent cells, this vector, combined with non-viral gene transfer vectors such as cationic lipids [28] and fusogenic liposomes based on Sendai virus [29,30], should constitute a useful tool for human gene therapy as well as provide on experimental model for gene therapy using small animals and for gene transfer experiments in non-primate cells. In addition, this study offers new insight into the mechanisms of the species-specific replication of EBV.

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