MODEST INCREASES IN THE TITERS OF HELPER VIRUS-FREE HERPES SIMPLEX VIRUS 1 (HSV-1) VECTORS BY PACKAGING IN A CELL LINE WITH INDUCIBLE EXPRESSION OF HSV-1 VP16 OR BY TREATMENT WITH N,N'-HEXAMETHYLENE-BIS-ACETAMIDE

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Summary. – A recently developed helper virus-free HSV-1 vector system has potential for both gene therapy and physiological studies, but relatively low vector titers have complicated use of this system. In this report, we explored improving the vector titers by isolating a Vero cell line that uses the tetracycline-regulated promoter system to induce HSV-1 VP16 at the beginning of the packaging procedure. We isolated the required cell line and demonstrated that it supports enhanced expression from a HSV-1 immediate early (IE) promoter. However, use of this cell line in helper virus-free vector packaging results in only a modest, ~2-fold increase in the vector titers. Additionally, treating the packaging cells with N,N'-hexamethylene-bis-acetamide (HMBA), which is known to induce HSV-1 IE promoters, also supports only modest increases in the vector titers. These results suggest that the lack of VP16 is not the rate limiting factor in the packaging procedure.

Key words: HSV-1 vector; helper virus-free packaging; HMBA; VP16

Introduction

HSV-1 vectors or amplicons (Geller and Breakefield, 1988) have a number of attractive features for gene transfer into quiescent cells to support both gene therapy and physiological studies. Thus, a growing number of investigators have used these vectors to modify neuronal physiology by expressing a wide variety of genes (for a review see Geller, 1999). However, use of a helper virus produces significant cytopathic effect (CPE) and an inflammatory response (Johnson *et al.*, 1992; Wood *et al.*, 1994). To eliminate essentially those side effects caused by the helper virus, we developed a helper virus-free packaging system for HSV-1 vectors (Fraefel *et al.*, 1996). A set of 5 cosmids that represents the HSV-1 genome (Cunningham and Davison, 1993) was modified by deletion of the cleavage/packaging sites. Cotransfection of this modified cosmid set and a HSV-1 vector into fibroblast cells results in the packaging of vectors into HSV-1 particles without production of the helper virus (Fraefel *et al.*, 1996). Production of helper virus-free vector stocks may be enhanced by the use of bacterial artificial chromosomes (BACs) that harbor the HSV-1 genome (Horsburgh *et al.*, 1999; Saeki *et al.*, 1998; Stavropoulos and Strathdee, 1998), although vector stocks prepared with the current HSV/BACs are contaminated with low levels of wild-type (wt) HSV-1 (Horsburgh *et al.*, 1999). Upon injection into the rat brain, helper virus-free vector stocks, prepared with the cosmids, produce a substantially weaker CPE and cell infiltration compared to that observed using helper virus systems

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Abbreviations: BAC = bacterial artificial chromosome; BHK = baby hamster kidney; CPE = cytopathic effect; DMEM = Dulbecco's Modified Minimum Essential Medium; HSV-1 = herpes simplex virus 1; HMBA = N,N'-hexamethylene-bis-acetamide; IE = immediate early; wt = wild-type; tet = tetracycline

(Fraefel *et al.*, 1996). Thus, this helper virus-free system addresses many of the problems with the previous helper virus vector systems, but its use is limited by relatively low titers.

VP16, the transactivator of IE promoters, is present in the HSV-1 virion and helps initiate HSV-1 infection (Roizman and Sears, 1993). In contrast, VP16 is not present at the beginning of helper virus-free packaging; the packaging is initiated by cotransfection of vector DNA and DNA(s) that represent the HSV-1 genome (Fraefel *et al.*, 1996; Horsburgh *et al.*, 1999; Saeki *et al.*, 1998; Stavropoulos and Strathdee, 1998). Thus, an approach to improving the titers is to provide either VP16 or HMBA, an inductor of IE promoters (McFarlane *et al.*, 1992) at the beginning of the packaging.

In this study, we derived Vero cell lines that use the tet-inducible promoter system (Gossen and Bujard, 1992) to regulate expression of HSV-1 VP16. Use of these cell lines in helper virus-free packaging supports only modest increases in the vector titers. Use of HMBA in the packaging also supports only modest increases in these titers.

Materials and Methods

Vectors pHSVlac and p4HSVlac (HSV-1 vectors) expressed *lac Z* gene from either the HSV-1 IE 4/5 promoter (Geller and Breakefield, 1988) or the IE 3 promoter (Smith *et al.*, 1995), respectively. Vectors pPURO and pTRE were obtained from Clontech. pTRE vector contained a tet-inducible promoter followed by a polylinker. The HSV-1 VP16 in 11 allele, which supports transactivation of IE promoters but may not be incorporated into HSV-1 particles (Ace *et al.*, 1988), was amplified by PCR (template: in 11 allele, 5′-primer: 5′-GGGTCTAGATTGTG GATACGAACCGGTGACGG-3′, the *Xba*I site underlined, complementary to nucleotides (nt) 103,540–103,563 (McGeoch *et al.*, 1988); 3′-primer: 5′-CCCGAATTCGATATCGTCT TTCCCGTATCAACCC-3′, the *Eco*RI site underlined, complementary to nt 105,087–105,111). The PCR products were digested with *Xba*I and *Eco*RI, and inserted into pTRE vector that had been digested with the same enzymes (pTRE-VP16in11).

Cells. 2-2 cells (Vero cells that express HSV-1 IE 2 (Smith *et al.*, 1992)), VTA11 cells (Vero cells that express the tet-off transcription factor (Ackland-Berglund and Leib, 1995)), and baby hamster kidney (BHK) cells were maintained in Dulbecco's Modified Minimal Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin, and 4 mmol/l glutamine at 37°C in a humidified incubator containing 5% CO_2 in air. G418 (0.5 mg/ml) was present during the growth of 2-2 cells but was removed 2 days before experiments. The modified Vero cell lines were derived and analyzed as follows. VTA11 cells, plated at 1 x 10^5 cells/ml and used 2 days later, were cotransfected with 3.6 μg of pTRE-VP16in11 and 0.36 μg of pPURO vectors using the calcium phosphate procedure (Graham *et al.*, 1974). Two days later, puromycin was added to the culture medium to final concentration of 10 μg/ml. After ~2 weeks, candidate clones were picked and expanded. A control transfection that used p4HSVlac vector yielded no colonies. Candidate cell lines (5 x 10^5 cells/60 mm Petri dish) were transfected with 2 μg of pHSVlac vector using the lipofectamine reagent. After 24 hrs, cell extracts were prepared, ß-galactosidase activity was assayed using o-nitrophenyl-D-galactopyranoside (Lim and Chae, 1989) at 420 nm. The ß-galactosidase activity obtained from a culture transfected with pUC19 vector was similar to that obtained from untransfected cells.

Helper virus-free packaging and titration of vector stocks. 2-2 cells or specific clones of VTA11 cells (1 x 10^5) were plated in 60 mm Petri dishes in 5 ml of DMEM with 10% of FBS, and the packaging was initiated 2 days later. For the packaging with specific clones of VTA11 cells, tetracycline (tet, at final concentration of 1 μg/ml) was added to the culture medium for 24 hrs prior to the cotransfection with the cosmid set /C6Δ**a**48Δ**a** (Fraefel *et al.*, 1996) and pHSVlac. The remainder of the protocol was similar to the described method (Fraefel *et al.*, 1996) modified by Sun *et al.* (1999) to improve the titers.

For packaging in the presence of HMBA (McFarlane *et al.*, 1992), 2-2 cells were treated with 5 mmol/l HMBA for either the first 24 hrs of the packaging or throughout the packaging (3 days). The remainder of the protocol followed the original one described by Fraefel *et al.* (1996). BHK cells were infected with the resulting vector stocks and 24 hrs later the standard staining was performed. X-gal-positive cells were counted under a microscope at the magnification of 10x and the titers (infectious vector particles (IVP) /ml) were calculated.

Results and Discussion

Effect of the induction of HSV-1 VP16 by the tet-off promoter system on the expression from a HSV-1 IE promoter

We investigated whether the induction of HSV-1 VP16 in Vero cells by the tet-off promoter can increase the activity of HSV-1 IE promoter. We used VTA11 cells, a Vero cell line that stably expresses the tet-off transcription factor (Ackland-Berglund and Leib, 1995). VTA11 cells were cotransfected with a vector that expresses HSV-1 VP16 from a tet-regulated promoter (pTREvp16in11) and a vector that expresses *lac Z* gene from the HSV-1 IE 3 promoter (p4HSVlac). After the transfection, the cells were incubated in either the presence or absence of tet, and the levels of ß-galactosidase activity were determined the following day. The experiment was repeated rwice with similar results. The results (Fig. 1) showed that the inducing of VP16 expression by removal of tet increased the IE promoter activity, as measured by elevated levels of ß-galactosidase activity. This effect required the vector that expresses VP16, and the magnitude of the effect increased with the amount of the vector Consistently with the properties of IE promoters (Roizman and Sears, 1993), we observed some ß-galactosidase activity also in the absence of VP16.

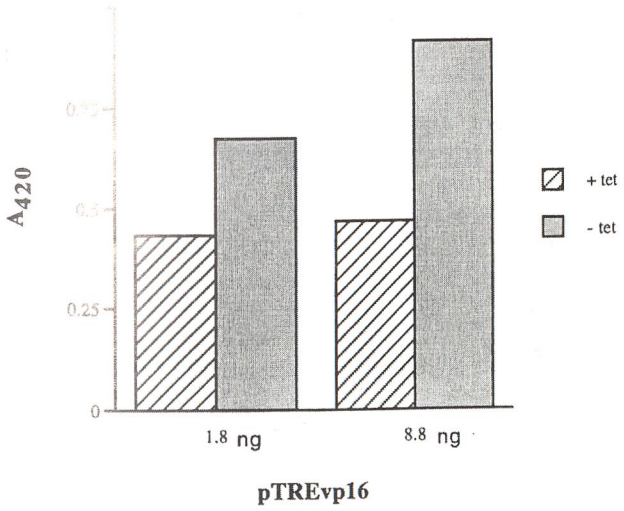

Fig. 1
Transient cotransfection of VTA11 cells with both pTREvp16 and p4HSVlac vectors results in tet-inducible expression of ß-galactosidase

VTA11 cells were cotransfected with the indicated amounts of pTREvp16 and 1 µg p4HSVlac vectors, incubated for 24 hrs in the presence or absence of tet, and then harvested. Each experiment was performed in duplicate, and the average values are shown.

Table 1. The levels of expression from p4HSVlac in candidate cell lines uninduced or induced to express VP16

Cell line	Transfected vector	ß-galactosidase activity[a]	
		+ tet	- tet
VTA11-10	P4HSVlac	0.71	1.02
VTA11-15	P4HSVlac	0.76	1.15
VTA11-22	P4HSVlac	0.83	0.96
VTA11-9	P4HSVlac	0.51	0.77
VTA11-14	P4HSVlac	0.76	0.78
VTA11-18	P4HSVlac	0.88	0.87
VTA11	p4HSVlac + VP16in11	–	1.03
VTA11	PUC19	–	0.02
VTA11	P4HSVlac	0.64	0.65

Candidate cell lines were transfected as described in Materials and Methods. Twenty-four hrs later, the cells were harvested and ß-galactosidase activity was assayed. At least 2 plates were assayed for each cell line in each experiment, each cell line was assayed in at least 2 independent experiments, and the means are shown.

[a]A_{420}/hr/4 x 10^5 cells.

Vero cell lines containing HSV-1 VP16 under the control of a tet-responsive promoter support the tet-regulated induction of HSV-1 IE promoter

To enable induction of HSV-1 VP16 prior to the transfection that initiates helper virus-free packaging, we derived cell lines that are stably transformed with pTREvp16in11 vector and can be induced to express HSV-1 VP16. VTA11 cells were cotransfected with pTREvp16in11 and pPURO vectors, and candidate clones were isolated by selecting for resistance to puromycin. To test the candidates for regulated induction of an IE promoter, each was transfected with p4HSVlac vector, incubated for 24 hrs in either the presence or absence of tet, and ß-galactosidase activity was measured. Four of the 30 cell lines that were tested exhibited tet-inducible increase in ß-galactosidase activity; a yield of ~10% inducible cell lines is typical for this system (Ackland-Berglund and Leib, 1995; Gossen and Bujard, 1992).

Table 1 shows the ß-galactosidase activity exhibited by these 4 cell lines and 2 cell lines that did not support the tet-inducible increase in ß-galactosidase activity. Each of these cell lines also exhibited a significant level of ß-galactosidase without induction, consistent with the activity of the IE 3 promoter in the absence of VP16 (Roizman and Sears, 1993).

Helper virus-free packaging in Vero cell lines that support an inducible increase in IE promoter activity

Three candidate cell lines were induced to express HSV-1 VP16, and 1 day later, they were cotransfected with

Table 2. Vector titers obtained from packaging in a cell line stably transfected with pTREvp16in11

Cell line	Tet	Vector titers, IVP/ml			Average induction[a]	Average increase vs VTA11 cells[b]
		Exp. 1	Exp. 2	Exp. 3		
VTA11-22	–	3.1 x 10^6	2.9 x 10^6	3.0 x 10^6	3.1-fold	1.9-fold
VTA11-22	+	1.5 x 10^6	1.3 x 10^6	6.0 x 10^5	–	–
VTA11	–	1.9 x 10^6	1.2 x 10^6	1.8 x 10^6	–	–

pHSVlac vector was packaged as described in Materials and Methods. The resulting vector stocks were titrated in BHK cells. At least duplicate plates were used for each titration, and the mean titers are shown.

[a]The titer obtained from packaging without tet divided by the titer obtained from packaging with tet, averaged over the 3 experiments.

[b]The titer from packaging in VTA11-22 cells without tet divided by the titer from packaging in VTA11 cells, averaged over the 3 experiments.

Table 3. Vector titers obtained from helper virus-free packaging in the presence or absence of HMBA

HMBA[a]	Vector titer, IVP/ml
None	1.5×10^5
1 day	3.4×10^5
3 days	3.0×10^5

pHSVlac vector was packaged as described in Materials and Methods. The resulting vector stocks were titrated in BHK cells. At least duplicate plates were used for each titration and the mean titers are shown.
[a]Five mmol/l HMBA was present either during the first day after the transfection or during all 3 days after the transfection, or was absent.

pHSVlac vector and the HSV-1 cosmids used for helper virus-free packaging. The resulting vector stocks were titrated in BHK cells. Of three cell lines, one consistently supported a 2- to 3-fold increase in the vector titers (Table 2).

Helper virus-free packaging in the presence of HMBA

On the basis of literature data on the positive effect of HMBA on the activity of HSV-1 IE promoters, we tested the effect of this substance on vector titers in our system. Following the transfection, addition of HMBA to the culture medium for either 1 or 3 days increased the vector titers ~2-fold (Table 3).

VP16 may not be the only tegument protein that enhances the vector titers obtained from helper virus-free packaging

We have previously reported (Sun *et al.*, 1999) larger, more than 5-fold increases in the vector titers by treating the packaging cells with previral DNA replication enveloped particles (PREPs, Dargan *et al.*, 1995) prior to the transfection that initiates the packaging. The PREPs supply essentially all the HSV-1 tegument proteins. Thus, because the PREPs support larger increases in the vector titers than those obtained by providing HSV-1 VP16 alone, these results (Sun *et al.*, 1999) suggest that both VP16 and additional tegument proteins play important roles in helper virus-free packaging of HSV-1 vectors.

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