An *oriP* expression vector containing the HIV-1 Tat/TAR transactivation axis produces high levels of protein expression in mammalian cells

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Abstract

A mammalian gene expression vector based on cytomegalovirus (CMV) enhancer/promoter (CMVe/p) for the regulation of gene expression was further optimized by adding *oriP* elements derived from Epstein-Barr virus (EBV) and the Tat/TAR transactivation axis from human immunodeficiency virus type 1 (HIV-1). Using the Tat/TAR-*oriP* expression vector, a transient transfection system was optimized for an extended culture period to produce large amounts of secreted IL-2SA (an IL-2 mutein) in HKB11 cells. We observed a 4-fold increase in IL-2SA expression in cells transfected with vectors containing the HIV-1 transactivation axis (Tat/TAR) or *oriP* elements alone when compared to cells transfected with the control vector having a CMVe/p. Cells transfected with expression vectors equipped with both *oriP* and Tat/TAR showed an 18-fold increase in IL-2SA expression. This transient transfection system maintained high secretion of IL-2SA for a period of 10-day with no appreciable loss in expression. We demonstrate that during this 10-day culture period, it was possible to produce 1–100 mg of proteins using 500 µg of plasmid DNA.

Introduction

Small amounts of protein (milligram quantities) can be made using various expression systems in yeast, microbial, insect, or mammalian cells. Due to secretion and glycosylation issues, mammalian cells remain to be the most desirable expression system. However, due to cell culture constraints, it requires a more involved process to produce and purify proteins in mammalian cells than in microbial systems.

To enhance expression in mammalian cells, viral enhancer elements and viral transactivator proteins such as immediate early protein 1 (IE1) from Cytomegalovirus (CMV) (Cherrington and Morcarski, J. Virol., 1989), E1a from adenovirus (Gilardi and Perricaudet, 1984), VP16 from herpes simplex virus (O'Hare and Hayward, 1984), BMLF1 protein (Lieberman et al., 1986; Kenney et al., 1989; Ruvolo et al., 1998) from Epstein-Barr virus, and Tat (trans-

activating protein) (Haseltine et al., 1998) from human immunodeficiency virus have been exploited. While such transactivation approach works well in short term transient transfections (3–5 days), in our experience, it is very difficult to derive stable high-expressing cells capable of producing high levels of protein over longer duration when employing simple viral transactivators.

To address the issue of high-level expression in transfected mammalian cells over periods longer than 3 to 5 days, we considered alternative strategies. We explored the use of the EBV *oriP* element and the EBNA1 protein (Yates et al., 1984; Sugden, 1987). *OriP* and EBNA function together to maintain input plasmid DNAs in an episomal state in most mammalian cells except rodent cells. A relevant observation has been that transfected DNAs with *oriP*/EBNA1 are more stably maintained than counterpart plasmids without these elements. Therefore, we reasoned that protein expression levels based on *oriP*/EBNA plas-

mids could be higher and more durable than alternative plasmid-based approaches. Here, we demonstrate the contributions of *oriP* and EBNA-1 with Tat/TAR from HIV-1 (Berkhout et al., 1989) to the expression of vectors which can produce high quantities (1–100 mg) of protein in transiently transfected mammalian cells over an extended period of 10 days.

Materials and methods

Cells

293EBNA (EBNA1 expressing 293 cells) was obtained from Invitrogen (Carlsbad, CA). HKB11 is a human somatic hybrid cell line (Cho, 2000) derived from the cell fusion of human embryonic kidney (293S) and a Burkitt's lymphoma origin, (2B8). 2B8 is a derivative cell line of HH514-16 (Rabson et al., 1983) which lacks the rearranged defective viral genome of P3HR1-EBV(P3HR-1) and does not produce EBV particles. HKB11 cells express EBNA1 from endogenous EBV.

Media

MICT7.0 is a proprietary protein-free medium supplemented with transferrin.

Plasmids construction

An *oriP* expression vector, pCEP4, consisting of *oriP*, EBNA1, and functional hph gene expression based on pBR322 was obtained from Invitrogen (Carlsbad, CA). Eighty-two base pairs of TAR element was prepared by polymerase chain reaction (PCR) using pBennCAT (Gendelman et al., 1986) as a template. Two primers were made by adding KpnI site at 5'-end of TAR and HindIII site at 3'-end of TAR. The resulting PCR product was digested with HindIII and KpnI and inserted into pCEP4 after digesting pCEP4 with KpnI and HindIII. The functional Tat gene expressing segment was prepared from pSVTAT (Peterlin et al., 1986) by digestion with BamHI and HpaI and subsequent modification with Klenow fragment. This fragment was inserted to pSV2neo after digesting with PvuII and HpaI. This functional Tat expressing segment was excised by digesting with PvuII and BamHI to insert into the other expression vectors as shown in Figure 1 (pSS185 and pSC221).

Transfection

Stationary transfections were performed using cells growing in anchorage dependent mode. Logarithmically growing HKB11 cells (1.5×10^6 cells) in 4 ml of fresh medium (MICT7.0) either with 5% fetal bovine serum (FBS) or without FBS were seeded in one well of a 6-well format tissue culture dish (Corning Inc. Corning, NY) and were allowed to attach to the bottom of the plate for two or more hours in a humidified CO₂ (5%) incubator at 37 °C. A cocktail (1 ml) consisting 5 µg of DNA and 20 µl of DMRIE-C reagent (Life Technologies, Rockville, MD) was prepared according to the manufacturer's protocol and was added to the well and mixed gently. The transfected cells were incubated in the CO₂ incubator at 37 °C.

Shaking transfections were performed using cells adapted to serum-free suspension conditions by rotating the culture on a shaker. Logarithmically growing HKB11 cells (5×10^6 cells) in 4 ml of fresh medium (MICT7.0) were mixed with one ml of the DNA/DMRIE-C complex as in the above stationary transfection. This 5 ml of transfected cells in a 6-well format was incubated on a shaker (90–100 rpm) in a humidified CO₂ incubator. Transfected cells were sub-cultured at 3 and 7 days post transfection (dpt) by splitting either 1:4 or 1:5 using MICT7.0 supplemented with transferrin and Pluronic F-68. The total culture volume was increased from the initial transfection volume up to 20-fold at 10 dpt. Tissue culture fluid (TCF) was analyzed by ELISA to determine the secretion levels of IL-2SA.

ELISA

For detection of IL-2SA, Dynatech Immulo-2 round bottom plates were coated with 1 µg/ml purified anti-human IL-2 (PharMingen, San Diego, CA) from rat using 0.1 M NaHCO₂ (pH 8.3) buffer, incubated at 37 °C for 3 hr in a humidified chamber, washed with PBS/0.05% Tween 20 (Sigma P-1379, St Louis, MO) and blocked with PBS/1% BSA for one hour at room temperature. Plates were then frozen in the blocking buffer PBS/1% BSA at –20 °C for short-term storage. Culture supernatants containing IL-2SA and purified IL-2SA (as a standard) were serially diluted with PBS/1% BSA (50 µl per well) and incubated at room temperature for two hours. Captured IL-2SA was detected using biotinylated anti human IL-2 (62.5 ng per ml; PharMingen, San Diego, CA) and HRP-streptavidin (125 ng per ml; Zymed Laboratories Inc. # 43–4323, South San Francisco, CA), followed

Figure 1. Physical maps of IL-2SA expression vectors used for the transfection study of the optimization of Tat/TAR-oriP related expression vectors. The individual element in each plasmid is follows. CMVe/p: cytomegalovirus derived, enhancer/promoter, MIS: a 5'-intronic sequence, TR: terminal repeat derived from Epstein-Barr virus (EBV), HSAG element (McArthur and Stanners, 1991), TAR: transactivating responsive sequence from HIV-1, Tat: transactivator gene from HIV-1, oriP: origin of plasmid replication derived from EBV, EBNA1: Epstein-Barr virus specific nuclear antigen 1, TKpA and TKp: poly A area and promoter, respectively, derived from function thymidine kinase segment, hph: hygromycin B phosphotransferase, SV40e/p: SV40enhancer/promoter, dhfr: dihydrofolate reductase. Note that the various elements described in the maps, MIS, TR, HSAG, and dhfr, showed no effects on IL-2SA expression in transient transfection assays.

by a one hour incubation each at room temperature. Substrate solution TMB (Kirkegaard and Perry Laboratories, Inc. # 50–7600, Gaithersburg, MD) was added and the reaction was stopped with 1 N HCl. The optical density was measured at 450 nm/570 nm using Molecular Devices Vmax kinetic microplate reader (S/N 04514, Sunnyvale, CA) and analyzed using the Softmax program provided by Molecular Devices. The IL-2SA concentration in the culture supernatant was determined by comparing to a standard curve of purified IL-2SA.

Results

Protein expression in transient transfections using Tat/TAR-oriP expression vectors

Mammalian expression vectors equipped with CMVe/p in a pBR322 plasmid direct relatively high level expression (1–20 μ g per ml) of heterologous proteins. However, some proteins such as IL-2SA are difficult to express using this approach. To see if we could improve upon a simple CMVe/p format, we constructed vectors which contain various enhancing elements, including those specifying for trans-acting proteins (e.g. Tat) and cis-acting replication element (e.g. *oriP*). These vectors were evaluated for the expression of IL-2SA in HKB11 cells, which express EBNA1 from endogenous EBV.

We found that the addition of *oriP* elements alone to a basic plasmid encoding IL-2SA did not drastically improve the synthesis of IL-2SA in transient transfections. We next constructed two expression vectors. The first contained an IL-2SA ORF in the pCEP4 vector (pSS179); the second incorporated the Tat/TAR transactivation axis into pSS179 (pSS185). To verify expression potency, both pSS179 and pSS185 were linearized with restriction enzymes to abolish *oriP* function. Five μ g of each DNA, in restricted and unrestricted plasmid formats, was used in transfections after confirmation of linearization and amount of DNAs by gel electrophoresis. Transfection was performed using the stationary transfection method in medium supplemented with 5% FBS. Expression levels of each plasmid were analyzed under the assumption that linearization of plasmids abolishes *oriP* function. As shown in Table 1, effects of *oriP* element (uncut-pSS179 versus cut-pSS179 and uncut-pSS185 versus cut pSS185) and Tat/TAR transactivation (cut pSS185 versus cut-pSS179 and uncut-pSS185 versus

uncut pSS179) on IL-2SA secretion were several folds higher (3–6 fold at 3 dpt) (summarized in Table 1). However, cells transfected with uncut pSS185 (intact Tat/TAR transactivation and *oriP* function) showed exceptionally higher levels (21-fold at 3 dpt) of IL-2SA expression when compared to cells transfected with cut pSS179.

We also tested other vector constructions containing the same functional elements at 3 dpt without modifying the plasmid DNAs with restriction enzymes to confirm the above results. Fold-increases of pSS225 (*oriP* vector) over pSL160N (non-*oriP* vector) and pSS185 (Tat/TAR-*oriP*) over pSS221 (non-*oriP*, Tat/TAR-vector) were 4.5 and 4.2, respectively. Fold-increase of pSC221 (non-*oriP*, Tat/TAR vector) over pSL160N (non-*oriP* vector) and pSS185 (Tat/TAR-*oriP*) over pSS225 (*oriP* vector) were 2.5 and 4.1 respectively. However, the fold-increase in IL-2SA was 18 (pSS185 over pSL160N) when both elements (Tat/TAR and *oriP*) were present. See Figure 1 for the physical maps of the expression vectors. Similar effects of *oriP* on the increase of IL-2SA titer were observed in HKB11 and 293EBNA cells transfected with pSC221 (Tat/TAR) and pSS185 (Tat/TAR-*oriP*) (Table 2). It was clear that Tat/TAR and *oriP* worked synergistically on the expression of IL-2SA as shown in Tables 1 and 2.

Shaking transfection using suspension culture

The transient transfection method was modified to establish efficient protein production in serum-free HKB11 cells. The optimal cell numbers per transfection using the same amount of transfection volume and materials (DNA and DMR1E-C) varied from 1.5×10^6 cells and 5×10^6 cells respectively for the two transfection methods employing stationary and shaking conditions. The shaking transfection method consistently sustained higher protein production in high-density cultures (Figure 3).

Protein production in prolonged transient transfections

In order to make larger quantities of proteins, we tested the transactivating proteins in long term cell cultures. Here, we evaluated several additional expression vectors of IL-2SA: pSC221, an IL-2SA expression vector incorporated with Tat/TAR transactivating elements; pSS225, an IL-2SA expression vector with *oriP* element, and pSS185, IL-2SA expression vector

Figure 2. Comparison of IL-2SA production in a prolonged and scaled-up transient transfection system using various expression-vectors under serum-free condition. The HKB11 cells transfected with $5 \mu\text{g}$ of each plasmid DNAs were sub-cultured by diluting 4 volumes at 3 dpt (5 ml + 15 ml) and again sub-cultured by diluting 4 volumes at 7 dpt (20 ml + 60 ml) before sampling for determination of IL-2SA titers. Bars represent individual titers of IL-2SA at the given day. The total yields (TY) of IL-2SA at 3, 7, and 10 days post transfection were calculated by multiplying the culture volume (ml) and titers ($\mu\text{g ml}^{-1}$). TT-oriP indicates Tat/TAR-oriP. The various expression vectors are dhfr (pSL160N), Tat/TAR (pSC221), oriP (pSS225), and Tat/TAR-oriP (pSS185), of which compared fold increase at 3 dpt was described in Table 1. Detailed plasmid maps are shown in Figure 1.

Figure 3. Comparison of stationary and shaking transfection using the same amount of pSS185 plasmid DNA and DMRIE-C. Transfection was performed as described in the methods and titer of the cultures was measured at 3 dpt. Titers of stationary transfection (open bars) and shaking transfection (filled bars) are from either individual transfection or side by side transfection (last three transfections).

Table 1. Comparison of fold increase in IL-2SA expression using cut and uncut *oriP* (pSS179) and Tat/TAR-*oriP* (pSS185) expression vectors and using various plasmid constructs

Plasmids	Titer ($\mu\text{g ml}^{-1}$) at 3 dpt	Fold increase		
		<i>oriP</i> effect alone (compared plasmid)	Tat/TAR effect alone (compared plasmid)	Tat/TAR + <i>oriP</i> effects (compared plasmid)
pSS179 (cut)	0.11			
pSS179 (uncut)	0.4	3.6 $\times$ (pSS179, cut)		
pSS185 (cut)	0.54		4.9 $\times$ (pSS179, cut)	
pSS185 (uncut)	2.37	4.4 $\times$ (pSS185, cut)	5.9 $\times$ (pSS179, uncut)	21 $\times$ (pSS179, cut)
pSL160N	0.28			
pSC221	0.64		2.5 $\times$ (pSL160N)	
pSS225	1.22	4.5 $\times$ (pSL160N)		
pSS185	5.08	4.2 $\times$ (pSS221)	4.1 $\times$ (pSS225)	18 $\times$ (pSL160N)

Detailed physical map of individual plasmid is shown in Figure 1. The description of individual vector-element is follows: pSS179, TAR-*oriP* vector; pSS185, Tat/TAR-*oriP* vector; pSL160N, non-*oriP* vector; pSC221, non-*oriP*, Tat/TAR vector; pSS225, *oriP* vector.

Table 2. Comparison of HKB11 and 293EBNA cells transfected with Tat/TAR (pSS221) and Tat/TAR-*oriP* vectors (pSS185) for IL-2SA secretion in serum-free medium at 3 dpt

Expression vector	HKB11		283EBNA	
	Titer ($\mu\text{g per ml}$)	Fold increase	Titer ($\mu\text{g per ml}$)	Fold increase
pSC221	0.50	1	0.62	1
pSS185	4.45	8.9	2.55	4.11

Note: Fold increase of IL-2SA by Tat/TAR and Tat/TAR-*oriP* was calculated by assuming IL-2SA production from pSC221 as 1.

with Tat/TAR transactivating elements and *oriP* element. HKB11 cells adapted to serum-free suspension conditions were transfected by the shaking transfection method. The control vector was pSL160N. The transfected cells were sub-cultured in serum-free medium by diluting 4–5 folds twice (e.g. 3 and 7 dpt) during a 10 day post-transfection period, which increased the initial culture volume by 20–25 folds. During this culture period, as shown in Table 1 (for 3 dpt) and Figure 2 (for 3, 7, and 10 dpt), the fold-increases of IL-2SA titers from cells transfected with pSS185 over those from cells transfected with pSL160N at 3, 7, and 10 dpt were maintained similarly, 18- (5.08 $\mu\text{g per ml}$ over 0.28 $\mu\text{g per ml}$), 20- (7.29 $\mu\text{g per ml}$ over 0.36 $\mu\text{g per ml}$) and 16- (5.76 $\mu\text{g per ml}$ over 0.36 $\mu\text{g per ml}$) fold, respectively. PSC221- and pSS225-transfected cells produced lower amounts of IL-2SA. The total yield of IL-2SA production from cells transfected with pSS185 (IL-2SA expressing vector with Tat/TAR and *oriP* elements) showed the highest yield ($\sim 470 \mu\text{g}$ of

IL-2SA from 5 μg of transfected DNA). This system was used to produce milligram-quantities of proteins, e.g. when 50×10^6 HKB11 cells (50 ml culture) were transfected with 50 μg of pSS185 plasmid DNA, we could obtain approximately 5 mg of protein at 10 days post transfection from a final culture volume of one liter. In our experience, the level of protein production from cells transfected with 500 μg of DNA in shake flasks was approximately 100-fold higher than cells transfected with 5 μg DNA in 6-well plates.

Protein expression under drug selection

Stable long-term expression of IL-2SA in HKB11 cells was evaluated using *oriP* vectors (pSS178 and pSS179) and Tat/TAR-*oriP* vector (pSS185) to see whether Tat/TAR-*oriP* effects could be maintained in a drug selection system in addition to the transient expression system. HKB11 cells cultured adherently in a 6-well format were first transfected with each of the three expression vectors using stationary transfec-

Figure 4. Tat/TAR transactivation effects of *oriP* expression vectors on IL-2SA secretion from HKB11 cells under hygromycin B selection. The transfected cells were maintained as described in the text, in which propagation of cells was done by performing two passages per week.

tion condition. Three days later, transfected cells were inoculated into a shake flask, 2×10^6 cells in 20 ml of fresh medium supplemented with $100 \mu\text{g ml}^{-1}$ of hygromycin B (HygB) and 5% FBS for drug selection. Cells were then sub-cultured twice per week using the same medium condition while maintained on a shaker. During the first three passages, cells were pelleted and resuspended each time into the same starting volume of fresh hygromycin B-containing medium. After this selection phase, cells were sub-cultured to maintain constant a cellular density of 5×10^5 cells ml^{-1} . Titers of IL-2SA production from each passage were measured by ELISA. Interestingly, eight weeks post-transfection pSS185-cells continued to maintain higher titers than cells transfected with either pSS178 or pSS179 (10-fold) (Figure 4). These results indicate that the Tat/TAR transactivation axis and the *oriP* element continue to work synergistically together (pSS185) under long-term drug selection. Although we observed that absolute expression levels of IL-2SA varied in three different independent experiments, the fold-increase of Tat/TAR-*oriP* (pSS185) over *oriP* (pSS179) was maintained similarly (6–10 fold) during the 8–10 weeks selection period. These data indicate that the Tat/TAR-*oriP* system is, particularly, optimal for low expressing proteins like IL-2SA. The mechanisms responsible for the elevated expression levels during the selection period are not known. When hygromycin B was removed from the culture media, the expression level of IL-2SA declined significantly in

2–3 weeks in all experiments. Addition of the TAR element-alone to the *oriP* expression vector (pSS178) produced no enhancement of IL-2SA expression over cells transfected with pSS179.

Discussion

With the advent of the new genomic age, there is a need to rapidly express unknown genes and to identify their biological functions. By incorporating the HIV transactivator Tat and its response element TAR (Berkhout et al., 1989; Haseltine et al., 1998) into an EVB-based *oriP* episomal vector (Yates et al., 1984), we have developed a transient expression system capable of producing 1–100 mg of proteins in a 10-day period. We have constructed various expression vectors (see Figure 1) to optimize this production system using IL2SA as a model gene.

As shown in Table 1 and Figure 2, there is a significant 4-fold increase in the expression of IL2SA in cells transfected with *oriP* vectors. The enhancing effect of Tat/TAR on IL-2SA expression is similar (3–6 fold) as *oriP* effect alone. Although Tsang et al. (2000) recently reported that Tat can enhance IL-2 production over 20-fold compared to a CMV promoter expressed in the absence of Tat, we should point out that such a calculation was based on a CMV promoter which lacked enhancer elements. Furthermore, there appears to be a synergistic effect on the expression of IL-2SA

when Tat/TAR and ori-P/EBNA were incorporated into a single vector. A significant 18–21-fold increase in the expression of IL-2SA was observed. The increase in IL-2SA expression was observed throughout the 10-day evaluation period. This transient production system was optimized for suspension cultures so that the production volume was increased to a culture volume of 10 l. This system has the advantage of using only 500 µg of DNA to transfect a seed culture (500 ml) and the culture is then scaled up to 10 l for harvesting within a 10-day period. This system is superior to the 3-day transient expression system where several mg (up to 10 mg) of DNA are required to transfect a 10 l culture.

We further demonstrated that the production of IL-2SA can be prolonged to an 8-week period (Figure 4) in the presence of hygromycin B selection. The level of IL-2SA remained high throughout the selection period. The productivity of cells transfected with pSS185 was consistently 6–10-fold higher than cells transfected with pSS179 and pSS178 (without TaT and TaT/TAR). It was of interest to note that the high expression of IL-2SA declined in 2–3 weeks after hygromycin B was removed from the culture media. It is not clear what is the operational mechanism behind this high-expressing production system.

In conclusion, we have developed a highly efficient large scale transient expression system. This system has the following salient features: (1) HKB11 cells, a unique cell line that can grow to high density (higher than 5×10^6 cells per ml) in suspension culture under protein-free conditions; and (2) an expression vector incorporated with *oriP* and Tat/TAR transactivating elements that show a synergistic effect on protein expression by maintaining high titer in the scaled up culture (20 folds of initial volume) over a prolonged time period (10 days post transfection). It is clear that the Tat/TAR-*oriP*/HKB11 system will find wide applications in the discovery of novel protein therapeutics.

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