Functional Analysis of OriS-flanking Sequences in Replication of HSV-1 based Amplicon Virions

Chenyang Jiang, Xinjing Liu, Qingzhi Wang, Ting Yang, Zhiqiang Han, Yuming Xu*

Department of Neurology, the First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, China
xuyuming@zzu.edu.cn

Abstract: The element of oriS as a necessary origin sequence for Herpes Simplex Virus type 1derived amplicon vector replicating consists of 45 bp oriS core region and flanking sequences. The flanking sequences of oriS located between gene US12/US1 and RS1 in HSV-1 genome were previously demonstrated to enhance oriS-dependent DNA replication, but the direct evidence of different oriS-flanking sequences in the replication of amplicon virions is still lacked. In this study, we performed a functional analysis of OriS-flanking sequences in replication of HSV-1 based amplicon virions by comparing the yields of virions packaged from amplicon vectors with various deletions of oriS-flanking sequences. The results showed that yields of amplicon virions were significantly reduced by deleting a AgeI/RsrII digested 181bp- fragment between the locus of 333bp-152bp upstream the oriS core region and a NcoI/StyI digested 314bp- fragment between the locus of 23bp-337bp downstream the oriS core region. Furthermore, deletion of a 87bp NcoI/SphI fragment at the locus of 250bp-337bp downstream oriS core region led to a dramatically decrease. Our study further demonstrated that oriS-flanking sequences played an important enhancement role in the amplicon vector replication evidenced at virion level.
[Chenyang Jiang, Xinjing Liu, Qingzhi Wang, Ting Yang, Zhiqiang Han, Yuming Xu. **Functional Analysis of OriS-flanking Sequences in Replication of HSV-1 based Amplicon Virions.** *Life Sci J* 2013:10(1):3132-3139]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 390

Keywords: Functional Analysis; OriS-flanking Sequences; Replication; HSV-1; Virion

1. Introduction

HSV-1 derived amplicon is a helper-dependent vector for gene delivery which is widely used in neuroscience for its low toxicity, large gene capacity, ease of manipulation and natural neurotropism (Spaete et al., 1982), mainly comprised of plasmid bone structure, multiple cloning sites for trans gene, and two cis-acting elements from the genome of HSV-1: the origin of replication (ori) and cleavage / packaging signal (pac) (Spaete et al., 1985). There are 3 origins of replication in HSV-1 genome, a single copy of oriL and two copies of oriS (Sandra, 2012), in which oriS located between the gene encoding ICP4 and ICP22/47 is commonly used to compose amplicon vector. The core region of oriS is a 45 bp unperfect palindrome comprised of a 17bp AT-rich sequence at its apex and two binding sites for the viral origin binding protein (OBP). The region flanking oriS between virus gene RS1 and US12 was believed to enhance the oriS-dependent DNA replication (Wong et al., 1991). Two regulatory elements adjacent to the oriS core region, named as Oscar L and Oscar R was believed to play a significant role and the deletion of them will reduce DNA replication by 90% (Nguyen-Huynh et al., 1998). However, those researches evaluated replication efficiency by transient plasmid DNA replication arrays, lacked of the functional analysis of the region flanking oriS in the replication of HSV amplicon virions. And a consistent phenomenon was observed in our previous work that amplicon vector constructed with oriS core region and both Oscar L and Oscar R but not other flanking sequence showed the efficiency less than 1% in generating amplicon virions while in previous study the replication was reported above 70% in the same situation (Nguyen-Huynh et al., 1998). In this study we made a functional analysis of the region flanking oriS to evaluate its function in the replication of HSV-1 amplicon virions and provide evidence for the optimization of amplicon vector.

HSV-1 amplicon DNA replicates in a head-to-tail rolling-circle manner and then is packaged into infectious amplicon virions with the helper genome providing necessary gene expressing (de Silva et al., 2009). Thus the quantification of the virions can be used as a direct viewing functional analysis to evaluate the replication efficiency of amplicon virions. It is difficult to separate amplicon virions from helper virus in helper-dependent packaging system. And the virions could not be quantified by the traditional titer measuring manner as causing no cytopathic effect after infecting the cells. Reporter gene such as red fluorescence gene Dsred can be used to make the infection of amplicon virions visible. Thus amplicon virions and helper virus can be titered simultaneously by quantifying cytopathic effect or fluorescence.

We developed a new method to generate bacteria gene free amplicon virions (mini-circle (Chen et al., 2003) amplicon, MC amplicon) with cre-loxp recombination system (Zhao et al., 2012). The replication of the mini circle amplicon virions is also helper dependent and shares the same process with

traditional amplicon virions. This kind of amplicon vector was also involved in this study. By previous work amplicon vector with oriS, the flanking intergenic region and the non-coding regions of viral immediate early gene US12 and RS1 was constructed. The roles of these two regions in the replication of amplicon virions were also investigated in this study.

In this study, amplicon vectors carrying red fluorescence gene were constructed with oriS core sequence and various parts of flanking regions. These vectors were packaged into infectious virions with viral proteins provided by wild type HSV-1 strain F and strain HF, and titered by TCID 50. The replication efficiency of these amplicon virions with different parts of oriS-flanking regions were compared during infection.

2. Materials and Methods

2.1. Cells, Plasmids and Virus

African green monkey kidney (Vero) and .293A cells were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The plasmids: (I) BAC-HSV-1-HF was constructed by our laboratory as reported before (Song et al., 2011). (II) pGM-T was stored by the laboratory. A replication-incomplete adenoviral vector system pAdv/BLOCK-iT™-DEST and pENTR™/U6 were purchased from invitrogen, USA. The multiple cloning sites (MCS) was designed by the author and synthesized by Sangon biotech co. ltd., Shanghai,China. All the plasmids were stored and prepared in the recombineering E.coli strain Top 10 purchased before and stored in our laboratory. The infectious HSV-1 strain F and strain HF was kindly provided by Dr William Jia from University of British Columbia Faculty of Medicine Neurosurgery,Canada.

2.2. Constructed amplicon vector with red fluorescent gene(Dsred) :

2.2.1. Constructed pGM-T-MCs-Dsred

A seuqence with restriction endonuclease sites ApaI/ AvaII /BglII /EcoRI /AgeI /SphI /SalI /HindIII /SpeI / KpnI / NotI / MluI was synthesized and was introduced into ApaI/MluI sites of pGM-T. Then the red fluorescent gene (Dsred) with a CMV promoter was introduced into HindIII/SpeI sites. The expression was tested in 293A cells after construction was finished.

2.2.2. Got segments containing oriS and various parts of flanking region from BAC-HSV-1-HF

Through previous work, we have got a 2.9kbp element comprised of (I)the core region of oriS (II)the intergenic sequence flanking oriS between US12 and RS1 gene.(III)the non-coding region of US12 and RS1 gene. The element was introduced into AgeI site of pGM-T-MCS with blunt ends for pGM-T-MCS-2.9kboriS. pGM-T-MCS-2.9kboriS was digested with a series of restriction nuclease for segments containing oriS core region and various parts of flanking region. These segments were named for the length and location of the parts in the flanking region, for example, an 1.2Kb oriS segment was get by Digesting pGM-T-MCS-2.9kboriS with EcoRI, this segment contained the oriS core region, 438bp sequence downstream(between the oriS core region and RS1 gene) , the whole intergenic region upstream and the non coding region of US12, so named it with oriS+438-US12. And an 816bp segment by digesting pGM-T-MCS-2.9kboriS With AgeI/Ecori containing 438bp sequence upstream and 333bp sequence downstream of oriS core region was named with oriS+438-333. In this way, by RSRII/StyI we got oriS+42-153, a 240bp segment containing the oriS palindrome and two oriS core-adjacent regulatory elements(Oscar L and Oscar R) (Figure 1) .A 560 bp segment oriS+271-254 was acquired by PCR with the primer 5'-TGTCCCTTTCCGATGCGA-3' and 5'-GGTGGTTTCCGCTTCCGT-3' before, and the 2.9kb segment was named oriS+RS1-US12, these five segments were introduced into AgeI site of pGMT-MCS-Dsred with blunt ends for pGMT-MCS-oriS-Dsred.

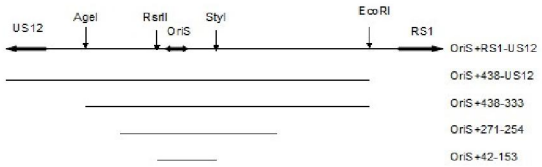

Figure 1: 2.9kb, 1.2kb, 816bp, 560bp and 240bp segments. The lines represent the segments with oriS core region and various parts of flanking region. The endonuclease sites, the core region of oriS and the non coding region of US12/RS1 are marked on the top line representing oriS+RS1-US12.

2.2.3. Constructed amplicon plasmid.

Cleavage and packaging signal (pac) has been acquired from "a" sequences in the 15bp TR region in BAC-TR as described before(Song et al., 2011). The pac signal was introduced into EcoRI sites of the pGMT-MCS-oriS-Dsred for pGMT-MCS-oriS-pac-Dsred, the amplicon plasmid (Figure 2).

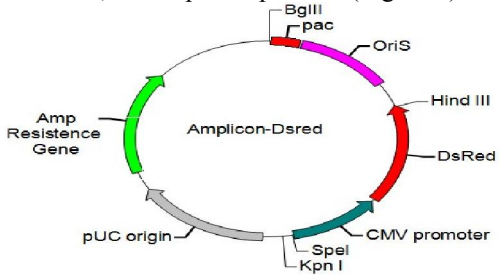

Figure 2: The amplicon plasmid.

2.3. Constructed of the vectors for MC amplicon system.

To test the oriS-dependent replication efficiency in mini-circle amplicon system, introduced the oriS-pac-Dsred (OPD) section of the amplicon plasmid described above between two loxp sites of pEnter vectors. Adv-loxp-OPD-loxp was acquired by LR recombination and replication-incomplete adenovirus was produced in 293A cells according to the methods reported before (Zhao et al., 2012).

2.4. Analyzed replication efficiency of amplicon and MC amplicon virions.

The amplicon and mini circle amplicon with oriS and various part of flanking region were divided into 4 groups in the functional analyze according to the types of vector and strains of helper virus used as in Table 1, and the tests for each group were repeated for 3 times.

Table 1：the groups of the functional test

groups	Amplicon type	Helper virus	Cells
A	Amplicon plasmid	HSV-1 strain F	293A
B	Amplicon plasmid	HSV-1 strain HF	293A
C	MC amplicon	HSV-1 strain F	VERO
D	MC amplicon	HSV-1 strain HF	VERO

2.4.1. Generation of amplicon virions.

All the plasmids mentioned above were stored and prepared in recombineering E.coli strain Top 10. After overnight culture, the plasmids were extracted by Axcygen Plasmids Extraction kits.

Vero cells and 293A cells were cultured in 10% serum DMEM complete medium. Before transfection, cells were transferred into 6-well plates at the rate of 2.5*10^6 per well and cultured in the CO_2 incubator at 37℃ over night. When the cells covered 70%~80% of each well under microscope, transfected the cells with 4 μg plasmids in each well according to the instruction of lipofectamine 2000.

The wild type virus HSV-1 strain F and strain HF were added to each well according to the groups with the MOI =0.1 as helper virus 24 hours after transfection , The cells were harvested 48 hours later and treated by repeated freezing-melting manner. The cell fragments were removed by centrifugation. The final solutions with helper virus and amplicon virions were stored at -80℃ and were marked as stocks R0.

2.4.2. Generation of MC amplicon virions.

MC amplicons were generated by co-infection of Adv-cre and Adv-loxp-OPD-loxp viruses into Vero cells with the Moi of 1, 24 hours later helper virus were added at MOI=0.1 after removing the old medium as described before (Zhao et al., 2012). The cells were harvested and treated in the same way as described in section 2.4.1 and marked as stocks MCR0.

2.4.3. Infected cells with stocks R0 and MCR0.

293A and VERO cells were cultured in 10% serum DMEM complete medium, and were transferred into 6 well plate at the rate of 2.5×10^5 cells per well before virus stocks added. Then 10μL of stock R0 or MCR0 was added into each well. The cells were harvested after 48 hours and treated in the same way as step 2.4.1., the final solutions was marked as stocks R1 and MCR1.

2.4.4. Titered amplicon virions and helper virus

Wild type helper virus can cause cytopathic effect (CPE) of the culture cells while the red fluorescence gene carried by amplicon virions can express after infecting the cells. Here we titered both the helper virus and amplicon virions of stocks R1 and R0 by method TCID50 based on gradient dilution. The mean value of the results from three repeated experiments was recorded as the final titer for amplicon virions or helper virus in each group.

2.5. Calculated the replication efficiency

Take the letter "a" representing the titer of amplicon virions or MC amplicon virions and "v" the titer of corresponding helper virus in stocks R0 or MCR0 and "A", "V" for stocks R1 and MCR1 with the unit TCID50/ml, during the infection of the stocks, we took the same amount of 10μl, meaning a/100 TCID50 amplicon virions and v/100 TCID50 helper virus were used to infect the cells in each well to get 2ml virus stock R1 or MCR1. Then the replication ratio C was calculated by the formula below:

$$C = \frac{A \bullet v}{a \bullet V},$$

3. Results

3.1. Viewing of the red fluorescence expressed by Dsred

Photographs of each group were taken under fluorescence microscope before the harvest of stocks R1 and MCR1, as presented in Figure 3 and Figure 4. From the figures: (I) The expressing of the trans gene Dsred at a very high level in both amplicon virions and MC amplicon virions with oriS+RS1-US12, oriS+438-oriS12 and oriS+438-333(marked as 2.9k oriS, 1.2K oriS and 816 oriS by their size in Figure 3 and Figure 4), and there were no significant differences among them from the horizons under microscope. (II)The replication of amplicon virions with oriS+271-254 and oriS+42-153 appeared lower efficiency judged by the number of red fluorescence points in several random horizons. (III) The control groups with plasmids or MC vectors without oriS appeared no red fluorescence.

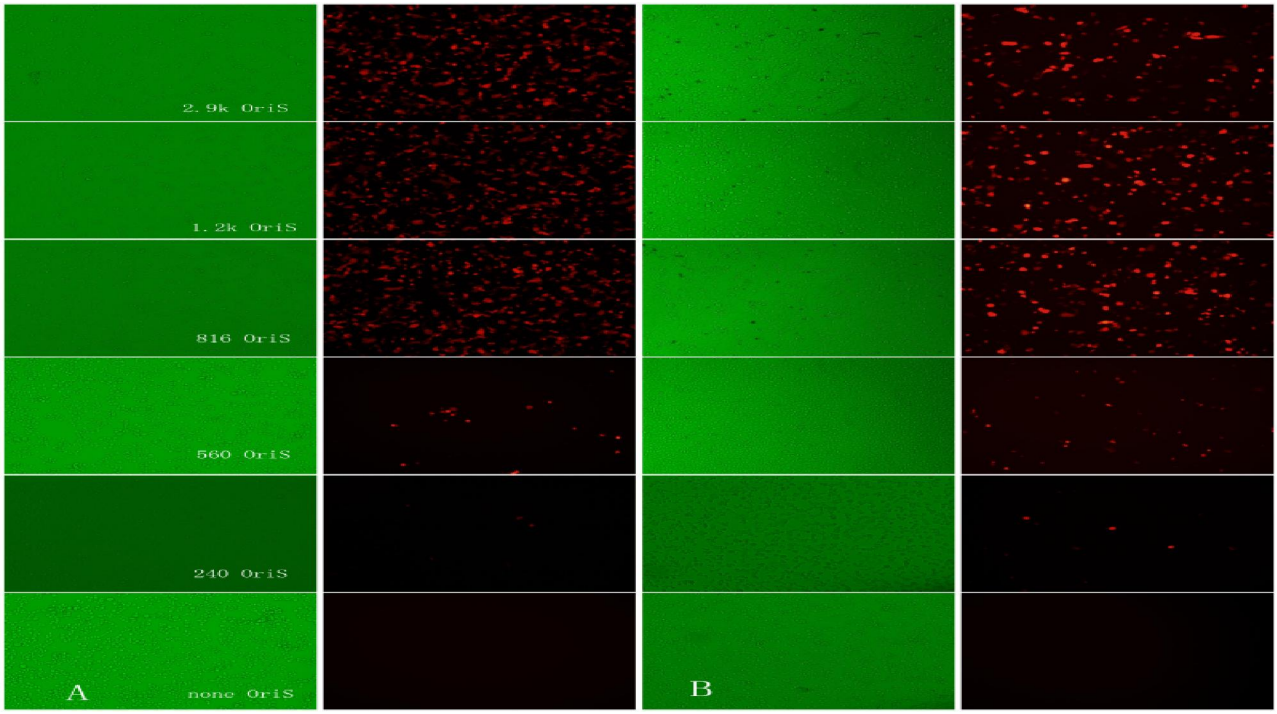

Figure 3: Red fluorescence of amplicon virions under microscope (100×)

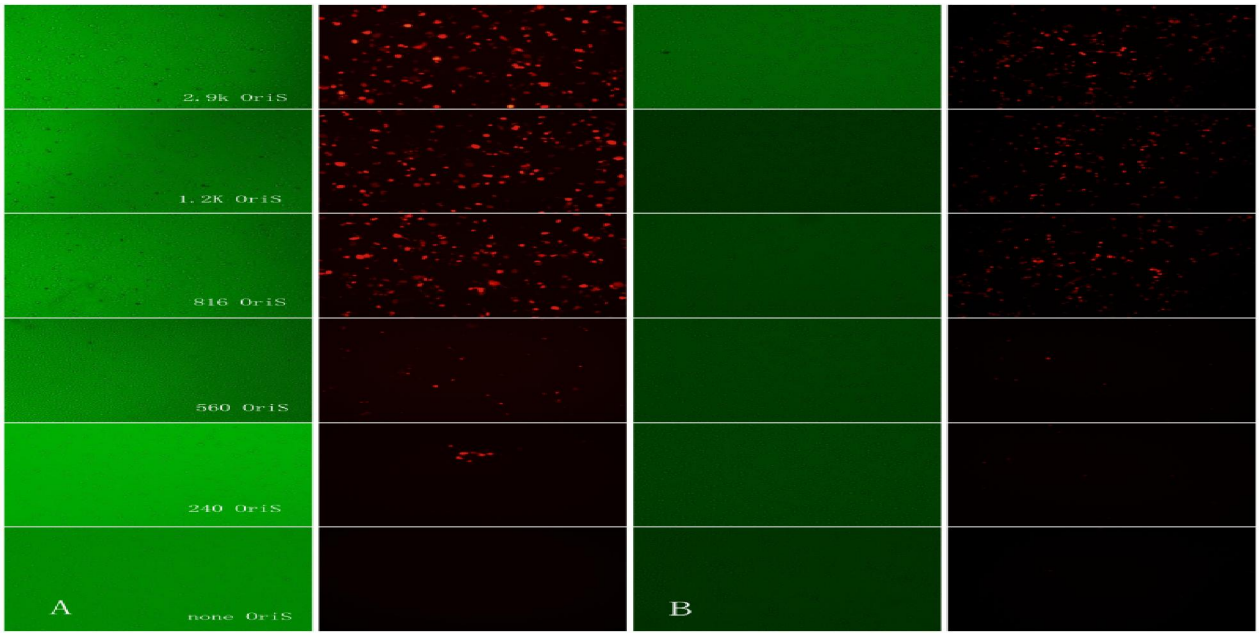

Figure 4: Red fluorescence of the MC amplicon virions under microscope (100×).

The photos in Figure 3 and Figure 4 each well were taken under both normal field (left) and dark field (right) under fluorescence microscope. Red fluorescence points were found in dark field. Figure 3 A and Figure 4 A presents the photos taken from groups with HSV-1 strain F as helper(group A and B) while 3 B and 4 B from groups with HSV-1 strain HF(Group C and D). Photos with the same oriS-containing segment are presented in the same lines and marked with the size of this segment, for example 2.9k oriS represents oriS+RS1-US12.

3.2. Titers of the virus and amplicon virions in each stock

The titers of amplicon virions and helper viruses were presented in Table 2 while the titers of MC amplicon virions and helper virus in Table 3, all the

titers reported were the mean value from 3 repeat experiments. The variation tendencies of the titers in all of the four groups were in accordance with the viewing in Figure 3 and Figure 4.

Table 2: The titers of amplicon virions and helper viruses (unit: TCID50/ml)

	R0(Group A)	R1(Group A)	R0(Group B)	R1(Group B)
oriS+RS1-US12	$1.47\pm0.92\times10^{7}$	$1.35\pm0.89\times10^{7}$	$1.02\pm0.69\times10^{7}$	$1.12\pm0.76\times10^{7}$
helper	$2.46\pm0.83\times10^{7}$	$1.47\pm0.93\times10^{7}$	$1.24\pm0.92\times10^{7}$	$1.01\pm0.22\times10^{7}$
oriS+438-US12	$1.41\pm0.95\times10^{7}$	$1.24\pm0.73\times10^{7}$	$1.05\pm0.54\times10^{7}$	$0.98\pm0.23\times10^{7}$
helper	$3.52\pm0.79\times10^{7}$	$1.89\pm0.45\times10^{7}$	$1.15\pm0.71\times10^{7}$	$1.11\pm0.47\times10^{7}$
oriS+438-333	$1.93\pm0.64\times10^{7}$	$1.94\pm0.69\times10^{7}$	$1.16\pm0.65\times10^{7}$	$1.20\pm0.52\times10^{7}$
helper	$3.68\pm0.77\times10^{7}$	$2.12\pm0.77\times10^{7}$	$0.99\pm0.45\times10^{7}$	$0.98\pm0.26\times10^{7}$
oriS+271-254	$4.37\pm2.13\times10^{5}$	$1.37\pm0.75\times10^{5}$	$1.93\pm0.97\times10^{5}$	$4.53\pm1.44\times10^{4}$
helper	$3.72\pm0.96\times10^{7}$	$3.77\pm1.09\times10^{7}$	$2.10\pm0.71\times10^{7}$	$2.32\pm0.76\times10^{7}$
oriS+42-153	$6.58\pm3.14\times10^{2}$	$4.59\pm2.21\times10$	$3.35\pm0.34\times10^{2}$	$0.85\pm0.22\times10$
helper	$5.34\pm0.97\times10^{7}$	$3.25\pm0.87\times10^{7}$	$3.68\pm0.46\times10^{7}$	$4.25\pm0.99\times10^{7}$

Table 3: The titers of MC amplicon virions and helper viruses (unit : TCID50/ml)

	R0(Group C)	R1(Group C)	R0(Group D)	R1(Group D)
oriS+RS1-US12	$8.25\pm3.12\times10^{6}$	$1.05\pm0.22\times10^{7}$	$7.63\pm3.27\times10^{6}$	$7.94\pm3.47\times10^{6}$
helper	$3.21\pm0.98\times10^{7}$	$3.30\pm1.71\times10^{7}$	$2.15\pm0.45\times10^{7}$	$2.03\pm0.39\times10^{7}$
oriS+438-US12	$7.46\pm2.07\times10^{6}$	$7.68\pm3.39\times10^{6}$	$6.10\pm5.43\times10^{6}$	$7.32\pm2.51\times10^{6}$
helper	$3.02\pm0.26\times10^{7}$	$3.42\pm1.02\times10^{7}$	$2.10\pm0.96\times10^{7}$	$2.43\pm0.44\times10^{7}$
oriS+438-333	$8.17\pm3.01\times10^{6}$	$9.92\pm6.42\times10^{6}$	$7.93\pm3.78\times10^{6}$	$8.15\pm8.23\times10^{6}$
helper	$3.34\pm0.72\times10^{7}$	$3.30\pm0.77\times10^{7}$	$2.30\pm0.47\times10^{7}$	$2.34\pm0.21\times10^{7}$
oriS+271-254	$1.34\pm0.36\times10^{5}$	$6.02\pm2.96\times10^{4}$	$1.02\pm0.90\times10^{5}$	$5.22\pm0.78\times10^{4}$
helper	$4.35\pm1.47\times10^{7}$	$4.43\pm1.01\times10^{7}$	$3.74\pm1.02\times10^{7}$	$4.35\pm1.02\times10^{7}$
oriS+42-153	$7.34\pm0.94\times10^{2}$	$8.23\pm1.23\times10^{1}$	$6.21\pm2.03\times10^{2}$	$3.23\pm0.44\times10^{1}$
helper	$6.05\pm1.22\times10^{7}$	$6.01\pm0.97\times10^{7}$	$4.33\pm0.77\times10^{7}$	$2.06\pm0.92\times10^{7}$

3.3. Replication efficiency of the amplicon and MC amplicon virions with different part of oriS-flanking region

The efficiency for each segments tested represented by replication ratio C according to the formula given in step 2.5.2 was calculated and given in Table 3 and Figure 6. The ratios of 715bp, 628bp and 414bp reported in Table 3 and Figure 4 were the result of further tests described in 3.4.

Table 4: replication ratio of the amplicon and MC amplicon virions.

	2.9kbp	1.2kbp	816bp	715bp	628bp	560bp	414bp	240bp
A	1.537	1.859	1.745	1.523	0.762	0.309	0.256	0.115
B	1.348	0.97	1.156	1.114	0.539	0.212	0.169	0.053
C	1.238	0.909	1.229	1.029	0.625	0.441	0.233	0.113
D	1.122	1.037	1.010	1.020	0.424	0.440	0.272	0.109

In this table all the segments tested were represented by its size. For example 816bp represents oriS+438-333. A, B, C and D represents the grouping shown in Table 1.

3.4. The further investigation of the different sequence between oriS+438-333 and oriS+42-153.

From the results above, the titers of amplicon virions and replication ratio decreased significantly when oriS+438-333 was substituted by oriS+42-153 in amplicon vector. To further investigate the function of the 576bp different sequence (396bp downstream and 180bp upstream) between these two segments, with a series of restriction endonucleases (NcoI, Sph1, BssHII and NruI) we got oriS+337-333 (715bp), oriS+250-333 (628bp) and oriS+167-202 (414bp) from oriS+438-333 (Figure 5). Amplicon and mini circle amplicon vectors were constructed with these segments and tested by the same methods described above. The results were shown in Table 3 and Figure 6 together with the former results.

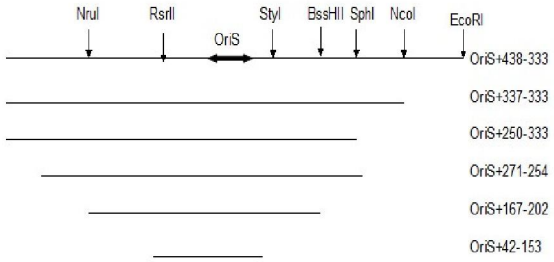

Figure 5: 715bp, 628bp, 414bp segments. oriS+271-254 (560bp) and oriS+42-153 (240bp) described in 2.2.2 is also shown in this figure. All the segments were represented by lines. The endonuclease sites and oriS core region are indicated on the top line representing oriS+438-333.

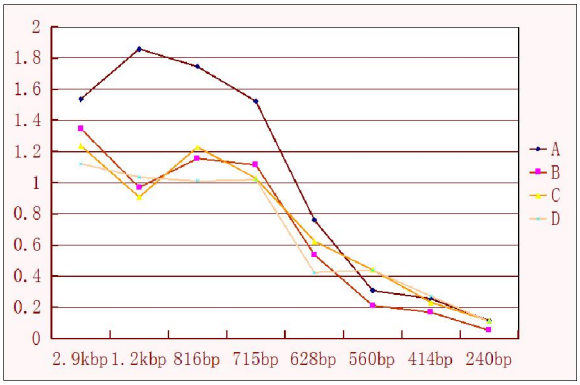

Figure 6: The variation tendency of replication ratio. A, B, C and D represents the grouping as given in Table 1, and the oriS-contained segments are labeled by their length, for example 2.9kb represents oriS+RS1-UL12.

3.5. The variation tendency of the replication ratio.

The replication ratio of amplicon virions showed similar variation tendency in the four groups although there were differences among the four groups due to the characters of the different packaging systems and strains of helper virus (Liu et al., 2011). Among amplicon virions with oriS+RS1-US12, oriS+438-US12 and oriS+438-333, no significant change of replication ratio and the titers of amplicon virions were detected, suggesting that the non-coding regions play no vital role in enhancing the replication of HSV-1 amplicon virions. oriS+438-333(816bp), oriS+337-333(715bp), oriS+250-333(628bp), oriS+271-254(560bp), oriS+167-202(414bp) and oriS+42-153(240bp) showed the correlation with the replication efficiency of amplicon virions, less flanking region led to lower efficiency. In which, the replication efficiency of amplicon virions decreased acutely when oriS+337-333 was substituted by oriS+250-333, suggesting the 87bp sequence located between 250bp and 337bp downstream of oriS core region played a significant role in enhancing the replication efficiency of HSV amplicon virions.

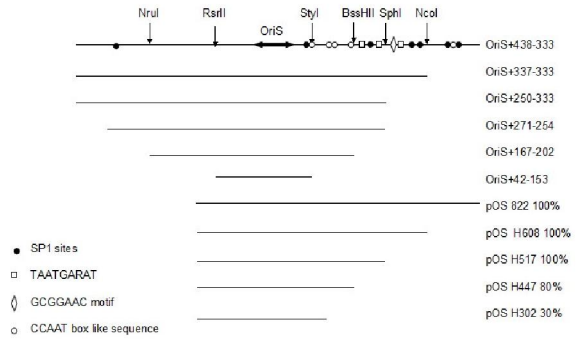

Figure 7: The elements in the region flanking oriS. The distribution of several already known elements in the 816bp intergenic region (oriS+438-333) are indicated on the top line, including SP1 binding sites, TAATGARAT, GCGGAAC motif and CCAAT box like sequence. pOS 822, pOS H608, pOS H517, pOS H447 and pOS H302 were the plasmids containing oriS and various parts of flanking region tested in previous research (Wong et al., 1991 and Nguyen-Huynh et al., 1998), the lines represent the segments containing oriS in these plasmids and the percentage represents the replication efficiency of each plasmid.

4. Discussions

From the results, the 87bp sequence located between 250bp and 337bp downstream of oriS core region played an important role in enhancing the replication of amplicon virions. This 87bp is a GC-rich region(77%) with following already known elements (Figure 7): (I)Two binding sites of Specificity Protein 1(SP1), a transactivator of gene expressing distributing in various cellular and viral genes (5'-(G/T)GGGCGG(G/A)(G/A)(C/T)-3') (Iwahori et al., 2007), (II)TAATGARAT as the binding site of HSV-1 transcriptional activator VP16 identified as an important transacting factor of the HSV-1 immediate early genes(Hughes et al., 1999). and (III)GCGGAAC motif required for the function of VP16 and efficient expressing of ICP4 as reported before (Triezenberg et al., 1988 and LaMarco et al., 1989). In which, the GCGGAAC motif is the only copy in the whole intergenic oriS flanking region, while there are another two copies of TAATGARAT elements located between endonuclease sites BssHII and SphI(+167 and +250). There are seven SP1 binding sites in the 816bp oriS+438-333 and the distribution was shown in Figure 5 and Figure 7. Two of them are located in the region between +337 and +438, the deletion of this

region caused no significant decreasing of replication efficiency according to Table 4 and Figure 6, while the deletion of the region between +167 and +250 led to obviously descending. This suggested that the TAATGARAT sequence together with GCGGAAC motif might play an important role in enhancing the oriS-dependent replication by stimulate the function of VP16.

According to current knowledge, VP16 plays an important role in the start of HSV-1 replication. When HSV-1 infect a cell, VP16 interacted with cellular factor Oct-1 and HCF to form a complex (Greaves et al., 1989 and Kristina et al., 1999). The complex then bind to the specific sequence TAATGARAT in virus genome and activate the transcription of the immediate early genes (ICP0, ICP4, ICP22, ICP27 and ICP 47), then the early genes and late genes start expressing in order and lead to the replication of HSV. The virus DNA formed a circle head-to-tail soon after entering the nucleus (Halford et al., 1996), then start rolling-circle replication after the expressing of a series of early genes, such as UL9 as the origin binding protein (OBP). Then the series-wound DNA product is cut into solo HSV-1 genome and packaged into virus (McGeoch et al., 1988). The replication of amplicon virions is believed as the same with HSV-1, sharing the gene expressing and regulation from helper genome. The difference is that the head-to-tail DNA is cut into segments of about 152kbp, containing one or tens of copies of amplicon genome according to the size of transgene (de Silva et al., 2009).

The conjecture that VP16 complex binding sites play an important role in enhancing the replication of amplicon virions can well explain the results of the functional analysis. According to the replication manner of HSV-1 and HSV-1 amplicon virions described above, the probable mechanism is that the binding of VP16 to TAATGARAT and GCGGAAC sites of amplicon DNA start an earlier and stronger expressing of the immediate early genes. VP16 is one of the tegument proteins of HSV-1 viral coat. It can enter the cell during infection and then serves as the transcription activator as talked above. Although there are several VP16 complex binding sites in the virus genome to activate the function of VP16, the extra binding sites may accelerate and enhance this process. Then the earlier and enhanced expressing of IE genes led to the enhanced the expression of other virus genes, starting replication of amplicon DNA earlier than the amplicon vector carrying no such binding sites in the same packaging system. And the replication may also start earlier than the helper virus genome DNA, leading to the superiority in competition with helper virus. As was reported in a previous study, in the helper-free amplicon packaging system based on cosmids (Fraefel et al., 1996), the titers of amplicon virions increased to 2 folds when low level VP16 was introduced into the cells 24 hours before the packaging components (Bowers et al., 2001). It demonstrated that VP 16 indeed played a role in enhancing the replication of HSV amplicon virions.

According to previous researches by transient DNA replication arrays, the DNA replication efficiency maintained almost the same until most of the region flanking oriS was knocked off. (Figure 7), and mutations introduced to VP16 binding sites caused no significant change (Wong et al., 1991 and Nguyen-Huynh et al., 1998). The different results were due to the methods in their studies, in which HSV-1 virus was added at 10 PFU per cell to start the replication of plasmids with oriS and flanking region, leading to the fast accumulation of VP16 which was clarified to suppress the replication of amplicon virions. (Bowers et al., 2001) And time for replication of oriS-containing plasmids was as short as 18 hours due to the rapid cell death. In this way the function of the VP16 complex binding sites in oriS-containing plasmids was covered. In our study, helper virus were added at a relatively low MOI (0.1 PFU per cell) according to former research on amplicon virions generation.(Wu et al., 1995), thus reduced the interference of virus genome.

As a transcription activator distributing in many cellular and viral genes, SP1 also act as the transcription activator of HSV immediately early genes and influence the DNA replication and functions by binding to the specific sites. (Kim et al., 2002) In a former study, knocking off the SP1 sites or CCAAT box like sequences both led to the decreasing of replication efficiency. In this study the results confirmed that SP1 sites and the CCAAT box like sequences might all play a role in the replication efficiency of amplicon virions although not as significant as the VP16 complex binding sites.

High replication efficiency is important for the generation of high titer HSV amplicon virions, thus the regulatory region flanking oriS is needed to enhance the replication efficiency. However in the practice of gene therapy with HSV-1 amplicon vector, less virus genome involved can bring about improved security. In this study, the 715bp oriS+337-333 is the shortest in those of replication efficiency higher than helper virus (replication ratio>1), thus being the optimum selection in constructing amplicon vector with current packaging system, both helper-dependent and helper-free. Higher replication efficiency is needed to increase the titer of amplicon virions because of the competition of helper virus or the low efficiency in these two kinds of packaging system (de Silva et al., 2009 and Fraefel et al., 1996). However, if

an advanced system providing more efficient helper-free packaging of amplicon virions can be developed in the future, the oriS core region together with less regulatory region flanking oriS, such as the 240bp oriS+42-153 clarified as maintain replication ability although in low efficiency, can be used in constructing amplicon vector.

In this study, we analyzed the function of the transcription regulatory region flanking oriS in the replication of HSV-1 amplicon virions. The region located between 337 bp upstream and 333bp downstream of oriS core region was clarified to enhance the replication of amplicon virions. A 87bp sequence located between 337bp and 250bp downstream of oriS core region was identified as of vital importance and VP16 complex binding site in this region was suggested to play the most significant role.

Corresponding Author:
Prof. Yuming Xu
Department of Neurology
the First Affiliated Hospital of Zhengzhou University
Zhengzhou, H450052, China
E-mail: xuyuming@zzu.edu.cn

References

1. Spaete RR, Frenkel N， The herpes simplex virus amplicon: a new eucaryotic defective-virus cloning-amplifying vector. Cell. 1982; 30(1):295-304.
2. Spaete RR, Frenkel N, The herpes simplex virus amplicon: analyses of cis-acting replication functions.Proc Natl Acad Sci U S A. 1985; 82(3):694-8.
3. Sandra K. Weller, Donald M.Coen, Herpes Simplex Viruses: Mechanisms of DNA Replication. Cold Spring Harb Perspect Biol. 2012; 4(9): a013011.
4. S W Wong, PA Schaffer, Elements in the transcriptional regulatory region flanking herpes simplex virus type 1 oriS stimulate origin function. J Virol. 1991; 65(5): 2601–2611.
5. Nguyen-Huynh AT, Schaffer PA,Cellular transcription factors enhance herpes simplex virus type 1 oriS-dependent DNA replication. J Virol. 1998; 72(5):3635-45.
6. de Silva S, Bowers WJ, Herpes Virus Amplicon Vectors, Viruses. 2009; 1(3):594-629.
7. Chen ZY, He CY, Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression in vivo, Mol Ther. 2003; 8(3):495-500.
8. Zhao J, Liu XJ, A novel approach for preparation of minicircle HSV amplicons by adenovirus mediated Cre-loxP recombination in mammalian cells, Life Science Journal 2012; 9(3):413-420.
9. Song B, Liu XJ, Han ZQ, Zhao L, Wang QZ, Lu JM, Xu YM, Construction of the HSV-1 strain HF amplicon and study on its unversal function between different HSV serotypes, Chinese journal of virology. 2011; 27(5):409-15.
10. Liu XJ, Song B, Lu JM, Wang QZ, Han ZQ, Xu YM, Construction of a recombinant BAC-HSV-1 strain HF with a GFP reporter gene and characterization of its infectious progeny virus, Chinese journal of virology. 2011; 27(3):238-43.
11. Iwahori S, Shirata N, Kawaguchi Y, Enhanced phosphorylation of transcription factor sp1 in response to herpes simplex virus type 1 infection is dependent on the ataxia telangiectasia-mutated protein. J Virol. 2007; 81(18):9653-64.
12. Hughes TA, La Boissière S, O'Hare P, Analysis of functional domains of the host cell factor involved in VP16 complex formation. J Biol Chem. 1999; 274(23):16437-43.
13. Triezenberg SJ, LaMarco KL, McKnight SL, Evidence of DNA: protein interactions that mediate HSV-1 immediate early gene activation by VP16. Genes Dev. 1988; 2(6):730-42.
14. LaMarco KL, McKnight SL, Purification of a set of cellular polypeptides that bind to the purine-rich cis-regulatory element of herpes simplex virus immediate early genes. Genes Dev. 1989; 3(9):1372-83.
15. Greaves R, O'Hare P, Separation of requirements for protein-DNA complex assembly from those for functional activity in the herpes simplex virus regulatory protein Vmw65. J Virol. 1989; 63(4):1641-50.
16. Kristina M. Johnson, Shahana S. Mahajan, Angus C. Wilson, Herpes Simplex Virus Transactivator VP16 Discriminates between HCF-1 and a Novel Family Member, HCF-2. J Virol. 1999; 73(5): 3930-3940.
17. Halford WP, Gebhardt BM, Carr DJ. Mechanisms of herpes simplex virus type 1 reactivation. J Virol. 1996; 70(8):5051-60.
18.McGeoch DJ, Dalrymple MA, Dolan A, Structures of herpes simplex virus type 1 genes required for replication of virus DNA. J Virol. 1988; 62(2):444-53.
19. Fraefel C et al. Helper virus-free transfer of herpes simplex virus type 1 plasmid vectors into neural cells. J Virol 1996; 70: 7190-7197.
20. Bowers WJ, Howard DF, Brooks AI, Halterman MW, Federoff HJ. Expression of vhs and VP16 during HSV-1 helper virus-free amplicon packaging enhances titers. Gene Ther. 2001; 8:111–120.
21. Wu X, Leduc Y, Cynader M, Tufaro F. Examination of conditions affecting the efficiency of HVS-1 amplicon packaging. J Virol Methods. 1995; 52(1-2):219-29.
22. Kim DB, DeLuca NA. Phosphorylation of transcription factor Sp1 during herpes simplex virus type 1 infection. J Virol. 2002; 76(13):6473-9.

3/3/2013