Generation of a recombinant herpes simplex virus which can provide packaging function for recombinant adeno-associated virus

WU Zhijian, WU Xiaobing* and HOU Yunde

State Key Laboratory for Molecular Virology and Genetic Engineering, Institute of Virology, Chinese Academy of Preventive Medicine, Beijing 100052, *China*

* *Corresponding author*

Abstract The generation of a recombinant HSV (rHSV) that can provide packaging function for rAAV production is described. A set of cosmids including cos48, cos28, cos6, cos14 and cos56, which represents the HSV-1 genome was used for generation of this rHSV. *Rep* and *cap* genes of AAV-2 were inserted into *Xba* I site of *UL2* gene on cos6, generating cos6-rcΔUL2. After being digested with *Pac* I, cos6-rcΔUL2 and the other 4 cosmids were cotransfected into BHK-21 cells. The recombinant virus HSV1-rc/ΔUL2 carrying *rep* and *cap* genes was generated due to the homologous recombination of the 5 cosmids. The results showed that the existence of *rep* and *cap* genes on this rHSV was stable from passage to passage and the rHSV could support the packaging of rAAV either in cells transiently transfected with AAV vector or in stable cell line harboring AAV vector. Further modification of this rHSV and optimization of conditions involved in rAAV preparation may lead to a large-scale production of rAAV in the near future.

Keywords: **recombinant AAV, packaging function, recombinant HSV.**

ADENO-associated virus (AAV) is considered to be an ideal gene delivery vehicle for human gene therapy due to its non-pathogenicity, ability to integrate into host chromosome in a site-specific manner and capacity of infecting postmitotic cells. The current strategy for producing recombinant AAV (rAAV) involves 2 recombinant plasmids. One is the expression vector harboring an exogenous gene expression cas-

sette flanked by the AAV inverted terminal repeats (ITR), which are necessary for viral replication and packaging. The other is the complementing plasmid (helper plasmid), which contains AAV genome with its 2 ITRs deleted. When host cells were transfected with these 2 plasmids and infected with adenovirus (Ad) to provide the helper function, rAAV particles could be produced[1]. However, this transfection method cannot support large-scale production of rAAV, therefore it cannot fulfil the demand of human gene therapy. Some investigators attempted to develop a recombinant Ad carrying *rep* and *cap* genes of AAV, which would provide all helper functions needed for rAAV production from an rAAV proviral cell line. Unfortunately, the development of such a recombinant Ad has met with failure, presumably because the AAV-2 Rep proteins inhibit Ad replication[2].

Herpes simplex virus type 1 (HSV-1) is another important helper virus for AAV. However, there has been no report for the generation of a recombinant HSV-1 carrying *rep* and *cap* genes so far. Recently, Conway *et al*.[3] and Shu *et al*.[4] reported the development of an HSV-1 amplicon stock expressing *rep* and *cap*. The stock is a mixture of HSV amplicon virus and wild type HSV helper virus, which can support rAAV replication and packaging. However, the ratio between *rep*/*cap*-expressing amplicon virus and helper virus in this stock was rather low and fluctuated from time to time, which makes the packaging effeciency unstable and limits its application.

In this note, we described the generation of a recombinant HSV-1 that can provide packaging function for rAAV production. The rHSV carrying AAV-2 *rep* and *cap* genes was generated by a new method we described previously[5], which was based on a set of cosmids that represents the whole genome of HSV-1.

1 Materials and methods

(ⅰ) Plasmids and bacteria. pSub201, the plasmid containing the complete AAV genome, is a gift from Samulski's lab[1] (University of North Carolina at Chapel Hill). pWAV-gfp is an AAV vector plasmid containing green fluorescent protein (GFP) gene. pCMV-lacZ-polyA is an eukaryotic plasmid expressing *E. coli lacZ* gene. Both of them were constructed in our lab. The plasmids above are propagated in *E. coli* DH5α. A set of cosmids that represents the whole genome of HSV-1 strain 17 was kindly provided by Davison[6]. This set of cosmids called set C is composed of cos6, cos28, cos14, cos56, and cos48, each of which contains part of the genome of HSV-1 strain 17. The cosmids are propagated in MAX Efficiency *E. coli* DH5α (GIBCO BRL).

(ⅱ) Cell culture. BHK-21 cell line was obtained from American Type Culture Collection (ATCC). 293 C18-EBgfp cell line was established in our lab by transfecting 293 C18 cell line with an EBV replicon-based vector, on which a GFP expression cassette under control of CMV promoter was flanked by the AAV-2 ITRs. The cell lines above were cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37℃.

(ⅲ) Construction of recombinant cosmids and generation of rHSVs. The 4.3 kb AAV-2 *rep* and *cap* genes under control of their native promoters were excised from pSub201 and inserted into *Xba* Ⅰ site of cos6. The recombinant cosmid was named cos6-rcΔUL2. The 4.5 kb CMV-lacZ-polyA cassette was cut out from pCMV-lacZ-polyA and inserted into *Xba* Ⅰ site of cos6, generating cos6-lacZ. Cos6-rcΔUL2, cos28, cos14, cos56, and cos48 formed set H cosmids. Cos6-lacZ, cos28, cos14, cos56 and cos48 formed set M. Set C, set H and set M cosmids were digested with *Pac* Ⅰ respectively and dissolved in TE solution after precipitating by ethyl alcohol. BHK-21 cells were transfected with the above DNA respectively using lipofectamine reagent (GIBCO BRL) according to manufacturer's instruction. 24 h after transfection, the mediums were replaced by RPMI 1640 supplemented with 2% fetal bovine serum. The mediums were replaced every 24 h until the cytopathic effect (CPE) appeared.

(ⅳ) Plaque purification of the rHSVs and their identification by PCR. When full CPE was observed, the cells were collected along with the medium, frozen and thawed 3 times. The lysates were diluted serially and then infected the BHK-21 cells growing on 6-well plates. After 3 d, 12 plaques that separated well from each other were randomly picked, seeded on BHK-21 cells growing on 12-well plates. 3 d later, cell lysate of each well was collected and preserved for identification. The recombinant viruses

were successively passaged on BHK-21 cells. Each passage was stored at −20℃ for use.

For preparation of DNA from recombinant viruses, the cell lysates were incubated in boiling water for 5 min. PCR was performed to detect whether the recombinant viruses contained *rep* gene. The upper primer is 5′-AAGGTGGTGGATGAGTGCTA-3′. The lower primer is 5′-TTGAAGGAGATGTATGAGGC-3′. The PCR product will be 321 bp in length. PCR for HSV1-*tk* gene was performed as HSV-positive control. The upper primer for HSV-*tk* is 5′-GCGTCTGCGTTCGACCAGGC-3′. The lower primer is 5′-GGAGCCAGAACGGCGTCGG-3′. The PCR product will be 395 bp in length.

(Ⅴ) Generation and detection of rAAV. BHK-21 cells were transfected with pWAV-gfp using lipofectamine reagent. 24 h post transfection, the cells or the 293 C18-EBgfp cells were infected with the above recombinant viruses respectively. The cells were collected after full CPE was observed. Following 3 freeze-thawing cycles and removal of cell debris by centrifugation, the cell lysates were heated at 56℃ for 60 min to inactivate the HSVs. BHK-21 cells growing on 24-well plate were infected with the above cell lysates. 24 h post infection, the cells were observed for the expression of GFP under an inverted fluorescence microscope.

2 Results

(Ⅰ) Generation of recombinant viruses. BHK-21 cells were transfected with *Pac* Ⅰ-digested set C, set H and set M respectively using lipofectamine reagent. The fragments of HSV-1 were excised from each cosmid by *Pac* Ⅰ digestion. Recombination occurred between the overlapping ends of these fragments. As a result, recombinant viruses could be generated within the transfected cells. 72 h after transfection, cytopathic effect (CPE) which indicated the productive infection of the virus began to appear in set C or set M-transfected cells. Full CPE was observed 5 d after transfection. The case was quite different in set H-transfected cells, in which CPE began to appear after 5 d and full CPE was observed 7 d after transfection. The delayed appearance of CPE in set H-transfected cells probably reflected the inhibition effect of Reps on HSV-1 replication.

(Ⅱ) Detection of *rep* gene in recombinant viruses by PCR. Using PCR analysis, both fragments of *rep* and *tk* gene can be detected in 12 samples of plaques from set H-transfected cells. In contrast, only *tk* gene can be detected in 12 samples of plaques from set C or set M-transfected cells. The result of PCR analysis (see fig. 1) was not changed using DNA of the fifth-passage virus as templates. The above results confirmed the existence of *rep* gene in rHSV obtained by set H transfection and indicated that the recombination rate was nearly 100%. The recombinant virus was named HSV1-rc/ΔUL2 (see figure 2).

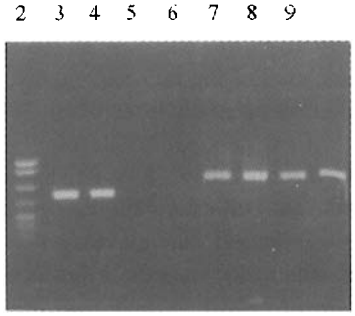

Fig. 1. PCR analysis for recombinant viruses. Lane 1, PBR322 digested with *Msp* Ⅰ as DNA marker; lanes 2—5, PCR for *rep*; lanes 6—9, PCR for *tk*. Lanes 2 and 6, First generation of HSV1-rc/ΔUL2; lanes 3 and 7, fifth generation of HSV1-rc/ΔUL2; lanes 4 and 8, recombinant virus obtained by set C transfection; lanes 5 and 9, recombinant virus obtained by set M transfection.

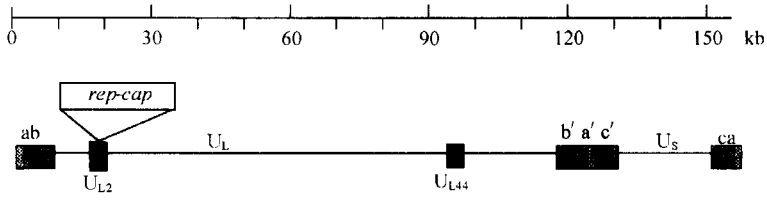

Fig. 2. Genome structure of HSV1-rc/ΔUL2.

(iii) rHSV carrying *rep* and *cap* genes can provide packaging function for rAAV. 1 mL HSV1-rc/ΔUL2 stock was used to infect 293/C18-EBgfp cells or BHK-21 cells which had been transfected with pWAV-gfp. Recombinant virus generated from set C was used as negative control. 72 h post infection, the cells were collected and lysed by 3 cycles of freezing and thawing. The lysates were used to infect BHK-21 cells. Approximately 1% of the cells infected with the lysates from HSV1-rc/ΔUL2-infected cells showed green fluorescence under an inverted microscope, which indicated the expression of GFP. No green fluorescence was seen in negative control group. The results showed that the HSV1-rc/ΔUL2 could support replication and packaging of rAAV. The rAAV that was generated from HSV1-rc/ΔUL2 infection has the ability to infect other cells.

3 Discussion

The helper functions of HSV-1 for AAV are different from those of Ad[3]. The Ad helper functions required for AAV replication are probably not involved in AAV DNA synthesis directly. Instead, the Ad helper genes make AAV replication possible through regulation of cellular gene expression and regulation of Rep expression. Ad can activate p5 promoter of *rep*, leading to expression of Rep. Rep in turn strongly inhibit the replication of Ad, which may be the main cause of the failure of the construction of Rep-expressing Ad vector. Unlike Ad, the early gene products of HSV-1 may be involved in AAV DNA synthesis directly. Although Rep also inhibits the replication of HSV-1, this inhibition is not as strong as that to Ad. Therefore, rHSV carrying *rep* and *cap* genes may be successfully generated.

The conventional method for construction of rHSV includes the insertion of foreign genes into the non-essential region of HSV-1 (such as *tk* gene) and the co-transfection of cells with infective HSV or its genome DNA. Homologous recombination takes place in cells and rHSV can be generated. However, the recombination rate of this method is rather low (between 10^{-4} and 10^{-1}). Considering the inhibition effect of Rep on the replication of HSV-1, the selection of rHSV containing *rep* and *cap* would be rather difficult if this method is used. In this study, a new strategy based on the recombination of HSV fragments which had been cloned into cosmids was utilized[5]. The recombinant HSV that carries *rep* and *cap* genes was generated conveniently, with the recombination rate much higher than that of the conventional method. The results showed that infective rAAV could be generated by infection of the cells containing rAAV vector with this rHSV.

In this note, generation of an rHSV that can provide all helper functions for rAAV peoduction is reported for the first time. In contrast to the mixture of wtHSV and amplicon virus carrying *rep* and *cap*, which had been recently reported by Conway *et al*.[3] and Shu *et al*.[4], the rHSV stock consists of a single kind of virus instead of a mixture. In addition, one rHSV particle carries one copy of *rep* and *cap* gene, which is superior to the amplicon system considering the fluctuation of the ratio of *rep* and *cap* to helper virus. Large-scale production of rAAV may be realized by infecting a stable cell line harboring rAAV vector with this rHSV.

Several reasons are responsible for the low production of rAAV in this study. First, the conditions for production of rAAV were not optimized, including the transfection rate, the state and copy number of vector DNA in vector cell line, the time and MOI of rHSV's infection. Second, the expression of HSV-1 ICP27 would interfere with the splicing of AAV Cap mRNA and therefore decrease the synthesis of Cap and influence the packaging of rAAV[3]. Third, the helper efficiency of HSV-1 to AAV may depend on the cell line employed[7]. HSV-1 was often as efficient a helper as adenovirus type 2 (Ad2) in generating infectious AAV particles in both KB and HeLa cells. However, in human embryonic kidney cells, AAV yield was substantially less using HSV-1 as a helper than that obtained using Ad2 as a helper. We are now establishing the AAV vector cell line using KB, HeLa and BHK cells respectively. Further efforts including improvement of this rHSV and optimization of the conditions involved in rAAV yield should be made to produce rAAV on a large scale.

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