Efficient \textit{in vivo} encapsidation of a shuttle vector into pseudo-simian virus 40 virions using a shuttle virus as helper

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We have designed shuttle vectors containing the late region of simian virus 40 (SV40) DNA (coding for the capsid proteins) which could be encapsidated into pseudo-SV40 virions during passage in monkey cells. We describe here the use of these shuttle viruses as helpers for the encapsidation of another shuttle vector into viral particles. Following cotransfection into monkey cells, the efficiency of encapsidation was similar for the shuttle virus and the other plasmid. The amounts of pseudo-SV40 virions recovered from the two vectors reflected the amounts of their DNAs present in monkey cells. Thus, the presence of the SV40 late region did not confer any significant advantage for encapsidation. The encapsidation of any shuttle vector into pseudo-SV40 virions is therefore possible and efficient, shuttle viruses constituting an interesting alternative to the use of SV40 as helper in this process.

The possibility of encapsidating shuttle vector DNA into pseudo-virions is of great interest since it would allow the introduction of the shuttle vector into cells, thus avoiding a transfection step. We designed plasmids containing the simian virus 40 (SV40) origin of replication (ori) and late region of its DNA (coding for the capsid proteins). These plasmids, designated shuttle viruses (Menck \textit{et al}., 1989), are able to replicate and to be encapsidated into pseudo-SV40 virions in monkey COS7 cells (SV40-transformed cells constitutively expressing the large T antigen; Gluzman, 1981). The PCF3A vector used in this study has been described (Madzak & Sarasin, 1991) and demonstrates the general characteristics of shuttle viruses. The production of infectious particles in the media from COS7 cells transfected with this vector was confirmed by finding extrachromosomally replicating plasmids in COS7 cells exposed to a 10-fold dilution of this medium (data not shown). The virus titre usually obtained after a lytic cycle of SV40-based shuttle viruses in COS7 cells was in the range of $10^5$ to $10^7$ p.f.u./ml (Menck \textit{et al}., 1987 and C. F. M. Menck, personal communication). The frequency of recombination between the shuttle viruses and the integrated SV40 sequences of COS7 cells, leading to the formation of wild-type SV40, was estimated to be less than $10^{-6}$ (Menck \textit{et al}., 1987).

This paper reports the use of these shuttle viruses as helper for the encapsidation of another plasmid into pseudo-SV40 virions during passage in monkey cells. We compared the encapsidation efficiency, after cotransfection into monkey CV1P cells, of two SV40-based vectors (Fig. 1). The vectors used were the pZ189 shuttle vector (Seidman \textit{et al}., 1985), carrying the SV40 early region and therefore coding for the large T antigen (necessary for the initiation of the replication of SV40-based vectors) and the PCF3A shuttle virus (Madzak & Sarasin, 1991), carrying the SV40 late region and therefore coding for the capsid proteins.

Our purpose was to determine whether both genomes could be encapsidated into SV40 capsids and to quantify the efficiency of this process, i.e. we wanted to determine whether the SV40 late region has some cis-acting role in the encapsidation efficiency of SV40-based plasmids.

The relative efficiencies of replication of the two vectors in monkey cells were initially established. We transfected separately or cotransfected (using the DEAE–dextran method; Wilson, 1978) equal amounts of PCF3A and pZ189 DNA into COS7 cells. After 3 days in culture, the extrachromosomal DNA was extracted and the ratio of the two plasmids determined by transformation of \textit{Escherichia coli}. Plasmid pZ189 carries an ampicillin resistance gene (Amp$^\beta$) and PCF3A a chloramphenicol resistance gene (Chl$^\beta$). Bacteria were transformed, using the method of Hanahan (1983), with an aliquot of the DNA preparation; half of the mixture was plated on an ampicillin-containing dish to screen colonies carrying pZ189, and half on a chloramphenicol-
containing one, to screen colonies carrying PCF3A. The numbers of bacterial colonies were determined and corrected for the respective transformation efficiency of each plasmid.

The PCF3A vector was found to exhibit a twofold growth advantage compared to pZ189 3 days after separate transfection or cotransfection into COS7 cells. Virus formation being undetectable after 3 days of culture, this advantage appeared to be due to plasmid replication in COS7 cells, and not to encapsidation.

The protocol used to compare the quantities of DNA and virus of the two plasmids after cotransfection (Fig. 2) was as follows. The two plasmids were first transfected into CV1P cells, the input pZ189:PCF3A DNA ratio being either 1:1 or 10:1. The total quantity of DNA transfected was kept constant, 0.5 μg per cell dish in each case. The replication of the PCF3A plasmid in CV1P cells is possible only if pZ189, supplying the T antigen, is present in the same cell. Seven days after cotransfection, the cell dishes were frozen and thawed three times to enable virus extraction. Subsequently, extrachromosomal DNA was extracted from the cells and the culture medium was kept as a virus stock.

Virus formation in CV1P cells occurs only when both pZ189 and PCF3A plasmids are present in the same cell. Quantification of this process was carried out

Fig. 1. Genetic maps of the shuttle vectors pZ189 and PCF3A. The two plasmids carry the SV40 ori, and a bacterial ColEl-type replication origin (derived from π mini-plasmids). Plasmid pZ189 carries the SV40 early region, coding for the large T antigen (TAg), and the AmpR gene. PCF3A carries the SV40 late genes, coding for the capsid proteins, the ChlR gene (CAT). The exact location on the SV40 sequence of the fragments used in these vectors is: clockwise from SV40 nucleotides 2533 (BamHI site) to 346 (HpaII site) for pZ189 (Hauser et al., 1986) and clockwise from SV40 nucleotides 5171 (HindIII site) to 2770 (BclI site) for PCF3A (Madzak & Sarasin, 1991; Menck et al., 1987, 1989). The location of these SV40 restriction sites is indicated on the maps by the name of the corresponding enzyme in parentheses. The suppressor gene supF is shown, as are the tryptophan promoter and the phage fλ (fλ ori) and M13 (M13 IG) origins of replication.

Fig. 2. Protocol used to compare the ratios of pZ189 and PCF3A vectors in the DNA and virus preparations recovered from monkey CV1P cells 7 days after cotransfection.
by infecting COS7 cells with the virus stock and extracting extrachromosomal DNA after 3 days. The pZ189:PCF3A ratio was then determined (by transformation of bacteria as described above) in the DNA preparation recovered from CV1P cells and in the DNA preparation recovered from COS7 cells (following infection with the virus stock recovered from CV1P cells).

Taking into account the twofold growth advantage of the PCF3A vector, the DNA preparation recovered from COS7 cells after 3 days in culture should reflect the composition of the virus stock recovered from CV1P cells. When a culture time of 3 days, instead of 7, was used for CV1P cells, no DNA was detected following infection of COS7 cells with the virus stock. This indicates that no detectable virus was produced 3 days after transfection and therefore supports the above conclusion that the twofold growth advantage of PCF3A after 3 days was due to replication and not to encapsidation.

The results are presented in Table 1 and demonstrate that when the initial cotransfection ratio was 1:1 the DNA yields were higher than with a 10:1 ratio. Indeed, the proportion of cells receiving the two plasmids was higher in the case of an input ratio of 1:1 and the virus production was therefore greater. However, the ratios of pZ189:PCF3A in recovered DNA and virus were lower than when a 10:1 ratio was used. Even with an initial cotransfection ratio of 10:1 in favour of pZ189, the amount of PCF3A DNA was of the same order of magnitude as that of pZ189 at 7 days post-transfection, despite the fact that pZ189 was able to replicate on its own in CV1P cells. This phenomenon could reflect a growth advantage of PCF3A (which is indeed the case, as set above) and/or more encapsidation of this vector. In this latter case, the pZ189:PCF3A ratio should be even lower in the recovered virus stock (which is not so).

Quantification of the virus recovered from CV1P cells needs to be corrected for the selective advantage of plasmid PCF3A which could occur during the 3 days of culture in COS7 cells necessary to obtain DNA from the virus stock. The pZ189:PCF3A ratio in recovered virus was 0.17 (with an input DNA ratio of 1:1). However, the correction for the twofold growth advantage of PCF3A during this period in COS7 cells raises this ratio to 0.34. Therefore, this difference between the vectors can be attributed to the higher amount of PCF3A DNA present in the CV1P cells when encapsidation occurred. A similar ratio was found in the recovered DNA (0.36). In the case of an input DNA ratio of 10:1 the pZ189:PCF3A ratio in recovered virus was 0.53. The ratio corrected for growth advantage was approximately 1. This is similar to the ratio of 0.72 in the recovered DNA. The proportion of the two plasmids in the virus stock corresponded approximately to that seen at the same time in the recovered DNA. Therefore, PCF3A does not seem to exhibit an advantage for virion formation. Moreover, a higher amount of pZ189 input DNA in cotransfection can compensate for the growth advantage of PCF3A, leading to an increase of the pZ189:PCF3A ratio in the virus stock.

We have shown that the presence of the SV40 late region does not confer a significant cis-acting advantage for encapsidation of the plasmid in viral particles. Hence, the use of shuttle viruses as helpers for the encapsidation of plasmids should be an efficient process, particularly when an initial excess of the plasmid of interest is used. Therefore, shuttle viruses could be used as an efficient helper in the preparation of virus stocks of a given shuttle vector. This method would enable mutagenesis studies avoiding a transfection step, or passage of the shuttle vector in cells for which transfection is inefficient, such as haematopoietic human cells. Introduction of DNA into such cells is required for gene therapy, and can be accomplished by encapsidation into non-pathogenic SV40 pseudo-virions (Oppenheim et al., 1986). Shuttle viruses could be advantageous compared to SV40, as they are unable to grow in target cells that do not provide the T antigen, nor do they produce this viral oncogene.

### Table 1. Quantification of the pZ189:PCF3A ratio in DNA and virus recovered from CV1P cells 7 days after cotransfection

<table>
<thead>
<tr>
<th>pZ189:PCF3A ratio in input DNA</th>
<th>DNA preparation from CV1P cells</th>
<th>DNA preparation from COS7 cells (virus from CV1P cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of bacterial colonies†</td>
<td>1:1 10:1</td>
<td>1:1 10:1</td>
</tr>
<tr>
<td>Chl R (PCF3A)</td>
<td>539000 14000</td>
<td>2900 750</td>
</tr>
<tr>
<td>Amp R (pZ189)</td>
<td>196600 10200</td>
<td>500 400</td>
</tr>
<tr>
<td>pZ189:PCF3A ratio</td>
<td>0.36 0.72</td>
<td>0.17 0.53</td>
</tr>
<tr>
<td>Ratio corrected for the growth advantage of PCF3A in COS7 cells†</td>
<td>0.34 1.06</td>
<td></td>
</tr>
</tbody>
</table>

* The DNA preparation was obtained from one 10 cm dish of CV1P or COS7 cells. Colony numbers were corrected for the respective transformation efficiencies of the two plasmids. Results were the mean of two independent cotransfections in CV1P cells.
† PCF3A exhibited a twofold growth advantage compared to pZ189 after 3 days in culture in COS7 cells. The corrected value reflects the ratio of the amounts of virus used in the infection of COS7 cells. The infection of one dish of COS7 cells (approximately 10^6 cells) was performed using 1/50 of the virus stock obtained from one CV1P cell dish. Considering a viral titre of 10^6 to 10^7 p.f.u./ml, the m.o.i. was low (10^-2 to 1 p.f.u. per cell).
Packaging constraints restrict the length of circular DNA that can be accommodated within SV40 capsids to between 70 and 105% of the length of SV40 DNA (5243 bp; Menck et al., 1990 and references therein). The two vectors used in this study are in this size range. However, shuttle viruses of various sizes have been used to analyse the constraints on SV40 encapsidation and have led to the observation that the optimal size (that remained constant through several lytic cycles and at which the vector was the least rearranged) was between 4 and 4.8 kb (Menck et al., 1990). These size constraints constitute a limitation to the use of SV40-based vectors for the transfer and expression of eukaryotic genes. The use of COS7 cells providing the T antigen for the pseudo-virion preparation would allow the encapsidation of vectors that do not code for this protein, and in which more space would be available for the gene(s) of interest. In fact, our results show that the SV40 early region present in the pZ189 vector was sufficient to promote its replication and encapsidation in monkey cells during coculture with a helper virus. We can therefore predict that a vector (for example an expression vector) can be propagated and encapsidated efficiently in monkey COS7 cells by using a helper virus, even if it contains only the small HindIII-HpaII SV40 restriction fragment (see the legend Fig. 1) surrounding the SV40 ori. SV40-based shuttle viruses offer an interesting alternative to the use of wild-type SV40 as helper virus in this kind of process.

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References


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