Virology 450-451 (2014) 122–131

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

The encapsidation of polyomavirus is not defined by a sequence-specific encapsidation signal

Hana Španielová *, Martin Fraiberk, Jiřina Suchanová, Jakub Soukup, Jitka Forstová

Department of Genetics and Microbiology, Faculty of Science, Charles University in Prague, Viničná 5, 128 44 Prague 2, Czech Republic

ARTICLE INFO

Article history:
Received 3 September 2013
Returned to author for revisions
25 September 2013
Accepted 10 December 2013
Available online 31 December 2013

Keywords:
Mouse polyomavirus
Encapsidation signal
Assembly
Pseudovirion
Packaging

ABSTRACT

Mouse polyomavirus (MPyV) is considered a potential tool for the application of gene therapy; however, the current knowledge of the encapsulation of DNA into virions is vague. We used a series of assays based on the encapsidation of a reporter vector into MPyV pseudovirions to identify putative *cis*-acting elements that are involved in DNA encapsidation. None of the sequences that were derived from MPyV have been shown to solely enhance the encapsidation of a reporter vector in the assay. The frequency of encapsidation strongly correlated with the total intracellular amount of the vector after transfection. The encapsidation of target DNA into the pseudovirions was shown to be non-specific, and the packaging of non-replicated DNA was observed. We propose that the actual concentration of target DNA at the sites of virion formation is the primary factor that determines its selection for encapsidation.

Introduction

Mouse polyomavirus (MPyV) and simian virus 40 (SV40) have historically served as molecular biology models and are considered potential gene therapy tools in anticancer therapy. MPyV seems to be superior for this type of application due to the absence of a pre-existing immunity in the human population. Both viruses are members of *Polyomaviridae*, which is a family of small, non-enveloped DNA viruses. The approximately 5.3-kb genome of MPyV circular dsDNA encodes three early regulatory proteins, designated small (ST), middle (MT) and large (LT) tumor antigens, and three late structural proteins, the viral proteins VP1, VP2 and VP3. Viral DNA is assembled with histones of cellular origin (except H1) in the form of a minichromosome and is encapsidated into an icosahedral capsid approximately 45 nm in diameter. The capsid consists of 72 capsomers composed of five monomers of the major capsid protein, VP1 and one of the minor proteins (VP2 or VP3), which faces the internal cavity of the viral shell (Chen et al., 1998). The processes of MPyV virion morphogenesis, genome selection and its encapsidation, albeit important for gene therapy vector design, are not well understood.

For SV40, detailed analyses of deletion mutants of the virus were performed and have revealed that the *cis*-acting DNA signal, which is important for the encapsidation of the SV40 minichromosome, resides in the enhancer region close to the origin of replication (ori) (Oppenheim et al., 1992; DalyotHerman et al., 1996). The signal is called *ses* (SV40 encapsidation signal). The recognition of the SV40 minichromosome during packaging is thought to be achieved via Sp1, a mammalian transcription factor, which binds *ses* within the regulatory region of the genome (Gordon-Shaag et al., 2002). Sp1 recruits the capsid proteins to the viral minichromosome by forming a recruitment complex that is composed of the Sp1 transcription factor and the SV40 capsomere (which is formed by five molecules of VP1 and one molecule of VP2 or VP3). Furthermore, the binding of the recruitment complex to *ses* via the association of VP2/3 with Sp1 leads to the repression of both early and late genes (Gordon-Shaag et al., 1998), thus regulating the transition from replication and transcription to assembly. Sp1 has not been found in mature virions (Roitman-Shemer et al., 2007), which indicates that before assembly, the transcription factor is displaced from the viral genome.

In contrast to SV40, the MPyV *cis*-acting signals and *trans*-acting proteins, which are prerequisites for the encapsidation of its DNA, have not been identified. The similarities in the organization of the regulatory region of both viruses justify the presumption that these elements exist and drive the virion assembly of MPyV. For example, the transcriptional regulator Ying-Yang 1 (YY1), which is a component of the nuclear matrix, has been found to directly interact with the major capsid protein VP1 of MPyV in vivo; however, the biological role of this interaction is not yet understood (Palkova et al., 2000). Three YY1 binding sites are present in the MPyV genome (Martelli et al., 1996, Gendron et al., 1996), and the direct interaction of VP1-YY1 could offer the possibility that YY1 can be involved in virion assembly similar to Sp1 in SV40 morphogenesis.

* Corresponding author. Tel.: +420 221 951 728, fax: +420 221 951 724.
E-mail address: hs@natur.cuni.cz (H. Španielová).

0042-6822/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.virol.2013.12.010

In this study, we attempted to identify the encapsidation signal for MPyV. Instead of deleting the viral genome and screening for the alleviation of genome packaging in mutants, we used methods for the efficient intracellular assembly of reporter vector (RV) DNA into pseudovirions, which have been recently developed for both SV40 (Oppenheim and Peleg, 1989) and papillomaviral vectors (Buck et al., 2004). We introduced parts of the MPyV viral genome in the target RVs to enhance their encapsidation. Moreover, by using the SV40 regulatory elements for MPyV encapsidation, we experimentally separated the process of encapsidation from MPyV DNA replication.

Table 1
Description of MPyV regions in reporter vectors.

Reporter vector	Region location (nucleotides)[a]	Landmarks
RV-1	4953–311	Regulatory region
RV-2	292–985	LT/MT/ST, intron
RV-3	971–1635	LT/MT
RV-4	1618–2180	LT
RV-5	2172–2836	LT
RV-6	2816–3496	poly(A) signal, VP1
RV-7	3479–4137	VP1
RV-8	4104–4741	VP2, VP3
RV-9	4748–3	VP2, enhancer

[a] MPyV genome numbering according to the GenBank database.

Results

Design and construction of pseudovirion-based assays

Based on SV40 research (Oppenheim et al., 1992), we reasoned that sequences around the origin of replication (ori) and the enhancer have the highest probability of containing the encapsidation sequence; however, we faced the problem that the importance of viral genome replication during virion assembly has never been systematically analyzed. For MPyV, it has been convincingly shown that the modification of the enhancer region can have an increased effect of one or two orders of magnitude on MPyV compared to SV40 DNA replication (Guo and DePamphilis, 1992) and that the manipulation of the viral genome in the YY1-binding area dramatically reduces genome replication (Spanielová, 2002). We assumed that the construction of an MPyV deletion mutant in a regulatory region would likely lead to restricted replication, decreased DNA copy number and limited encapsidation, regardless of its intrinsic packaging potential. For this reason, we did not use the deletion mutant approach, as performed during the identification of *ses* (Oppenheim et al., 1992). Instead, we developed several assays based on pseudovirion technology. This technology allows for the generation of pseudovirions consisting of capsid proteins and an RV (instead of a viral genome) associated with cellular histones in producer cells.

The first assay, called the Reporter vector Encapsidation into Pseudoivirions system (REPs) is designed to use the pseudovirions for the subsequent experimental infection, which causes the transduction of the reporter gene. The efficacy of the pseudoinfection (transduction) by pseudovirions, which contain different RVs (measured by the activity of the reporter gene product after the gene transduction by each individual vector), then corresponds to the efficacy of their packaging. The RVs that carry the part of MPyV with a putative encapsidation signal sequence would be transduced with the highest frequency. Specifically, the system consists of a series of RVs that serve as target DNAs for encapsidation, helper vector(s) providing the capsid proteins in *trans* and two cell lines: one allowing the production of pseudovirions (producer cells) and the second (detection cells) allowing the sensitive screening of reporter activity after pseudoinfection (transduction).

RVs were constructed from the pGL3-Control vector (Promega), which harbors the luciferase reporter gene under the control of a strong SV40 promoter. The vector also carries the SV40 enhancer and SV40 ori, which ensures the replication in monkey and human cell lines with the constitutive expression of the SV40 large T-antigen. The plasmid pGL3-Control does not contain any MPyV-derived sequences. The plasmid's size corresponds to the size of the MPyV genome (5.3 kb); thus, it serves as the control RV throughout the study. For the construction of RVs, the MPyV genome was divided into nine 650-bp-long fragments that cover the entire viral genome (Table 1), and each fragment was cloned into the multiple cloning site of the shortened pGL3-Control plasmid (pGL3-ΔC, see the section "Materials and methods"). The final size of all RVs was approximately the size of the MPyV genome to allow the unrestrained encapsidation of constructs.

The helper vectors ph2-VP1 (encoding VP1 and VP2) and ph3β (encoding VP3 and β-galactosidase) were constructed from codon-optimized MPyV structural genes (see the section "Materials and methods" for detail). Both helper vectors contain the SV40 origin of replication, and their size exceeds the MPyV genome size by 2.5 kb to prevent their encapsidation. The β-galactosidase gene was included in the system to normalize the transfection efficiency. In some experiments, the pCG-VP1/2/3 vector (6.5 kb) that encoded all three structural proteins under the control of the MPyV late promoter was used. The descriptions of all of the plasmids that were used in the study are shown in the Supplementary Table S2. Human embryonic kidney 293TT cells that stably expressed the SV40 large T-antigen to enhance the replication of SV40 origin-containing plasmids were used as producer cells.

The second assay, called the qPCR assay, is derived from the REPs system. During the qPCR assay, the RVs are isolated directly from pseudovirions and are used for quantitative PCR (qPCR) analysis to determine the most frequently encapsidated RV. This assay eliminates the need to determine the efficacy of packaging by secondary transduction to the screening line, which can be a potential confounding factor. Moreover, due to sequence-specific detection of each RV, it allows direct identification of RV from a mixture of pseudovirions carrying different RVs. There is, however, a potential disadvantage in that fragmented DNA, or DNA of less than unit length, enclosed in so called "empty" capsids can be detected.

The third assay, called the transformation assay, also directly identifies encapsidated RV, but the DNA extracted from pseudovirions is used for the transformation of bacteria. The identification of encapsidated RVs is based on the subsequent sequencing of vectors isolated from bacterial colonies. In contrast to the qPCR assay, this approach ensures the identification of full-length intact encapsidated vectors, thus further increasing the accuracy of the experimental evaluation.

Pseudovirions are generated inside the cell nucleus

To ensure that MPyV pseudovirions can be successfully generated in cells using our system, we co-transfected 293TT producer cells with helper plasmids, ph2-VP1, ph3β and the reporter control vector, pGL3-Control. The transfection efficiency was evaluated by immunofluorescence. Capsid proteins and luciferase genes were coexpressed in 87% of transfected cells (data not shown). At 48 h post-transfection, cells were collected and were then either subjected to a purification procedure or processed directly for electron microscopic examination on ultrathin sections in order to exclude a theoretical possibility that transducing particles are nonspecifically formed in the cell lysate through a self-assembly process.

Electron microscopy confirmed that viral particles could be purified from cell extracts (Fig. 1A) and that the cell-associated viral particles could be detected inside the nuclei of transfected cells (Fig. 1B).

A region of early introns (nucleotides 292–985) in the MPyV genome surpasses other regions in the REPs assay

The REPs assay was designed as a rapid screen for packaging signal. The pseudovirions, which were formed after the transfection of producer cells with helper vectors and with each individual target RV, were not purified, but the whole-cell lysate that was treated with DNase I was used for the transduction of the detection cell line. In this way, the efficiency of the encapsidation of a given RV in producer cells can be estimated from the normalized activity of luciferase that was measured in the corresponding cell extract from transduced detection cells. Initially, MPyV-permissive mouse 3T6 fibroblasts were used as the detection cell line. Using these cells, we failed to detect any luciferase activity in the REPs assay (data not shown). However, high luciferase activity was achieved in COS-1 cells after the transduction of pGL3-Control. COS-1 cells that express the SV40 large T-antigen support the replication of plasmids containing the ori. We concluded that (i) the replication of the RV in detection cell lines is necessary to increase the sensitivity of the assay and (ii) RVs can

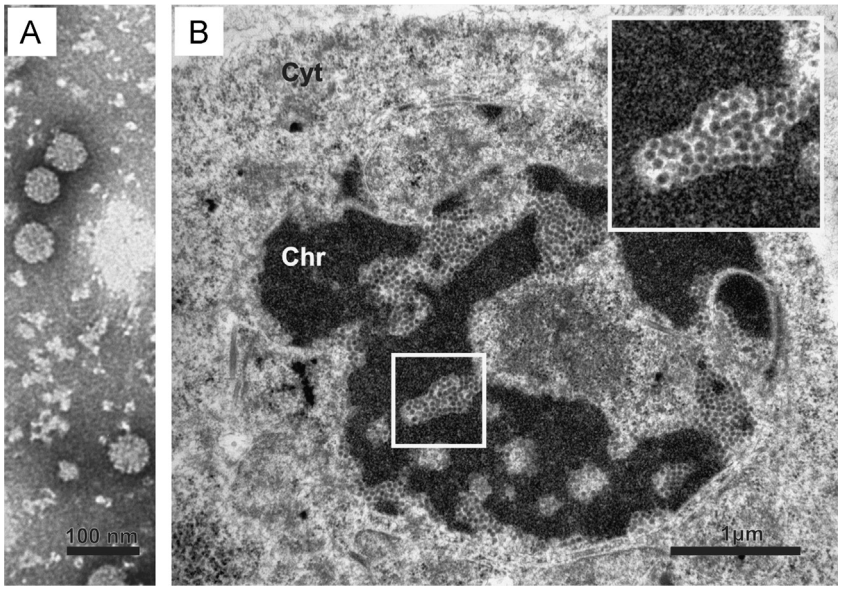

Fig. 1. Electron micrographs of MPyV viral particles that were produced in 293TT cells. (A) Purified viral particles were visualized by negative staining. (B) For transmission electron microscopy on ultrathin (70 nm) resin sections, cells were fixed at 48 h post-transfection. Particles were observed in clusters (as shown in the inset) inside the nucleus in the periphery of the condensed chromatin (Chr). Cyt, cytoplasm.

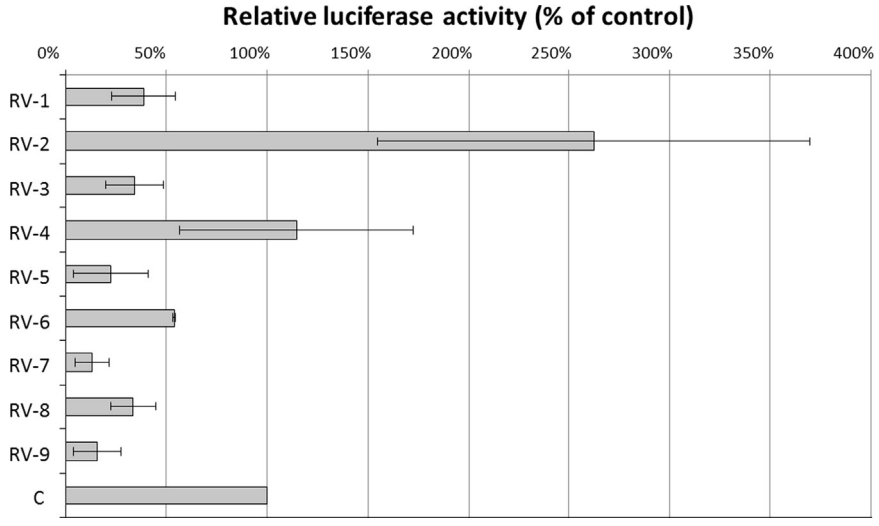

Fig. 2. The REPs assay in COS-1 cells. The 293TT cell line was co-transfected with the helper vectors ph2-VP1 and ph3β and each of the indicated RVs. A cell lysate from the production cell line was used for the transduction of the COS-1 cell line. Luciferase activity was measured in cell lysates that were prepared at 48 h post-transfection and in lysates that were prepared at 48 h post-transduction. Relative luciferase activity was determined for each cell lysate as described in the section Materials and methods, and the final relative luciferase activity was calculated by dividing the relative luciferase activity that was determined in COS-1 cell lysates with the relative luciferase activity that was determined in the corresponding 293TT cell lysates. The graph shows the results of three independent experiments, and the relative luciferase activity that was calculated for each sample is expressed as a percentage of the relative luciferase activity that was determined for the pGL3-Control (C) vector (represented by 100% on *x*-axis). The standard deviations of the mean are indicated by error bars.

be effectively transduced by MPyV pseudovirions. Finally, the COS-1 cell line was employed as a detection cell line in REPs assays.

The results from three separate REPs assay screens are shown in Fig. 2. Out of all RVs, reporter vector 2 (RV-2) was the only vector that was transduced into the COS-1 cell line more efficiently than the control vector. This observation suggested that the RV-2 MPyV genome region (nucleotides 292–985) could enhance the packaging of DNA by MPyV capsids. However, we noted that the transfection of RV-2 always resulted in the highest luciferase activity in producer cells (data not shown). This result may indicate that this MPyV region can convey another quality, such as replication enhancement or plasmid stability, instead of the putative packaging signal. Although this bias was corrected by normalizing the results using β-galactosidase activity in cell lysates used for transduction, the bias could not be avoided during the final screen in the detection cell line that supported replication. For this reason, we decided to verify the data by an independent method and to focus on RV-2 and its unique qualities.

None of the MPyV regions are required for the encapsidation of the reporter vector into the pseudovirions

We decided to trace encapsidated RVs directly in assembled pseudovirions by qPCR assay. We mixed all RVs (including the pGL3-Control) together with helper vectors (ph2-VP1 and ph3β), transfected the entire mixture into the 293TT producer cells and separately analyzed the DNA contents of the pseudovirions and the total extrachromosomal DNA in transfected cells. The transfected producer cells were harvested at 48 h post-transfection and divided; one-half was used for the extraction of total extrachromosomal DNA (by a modified Hirt method), and the second half was used for the extraction of nuclease-resistant (encapsidated) DNA. Both samples were used for qPCR with primers that were specific for each RV. The encapsidation efficiency for each RV was determined as the ratio of the amount of the RV that was detected in the nuclease-resistant pool and in the total extrachromosomal DNA. In two separate experiments, none of the MPyV regions were able to significantly increase the encapsidation efficiency of any RV over the control vector (Fig. 3B). Surprisingly, RV-1, which possessed a complete set of MPyV regulatory elements that included the MPyV ori, repeatedly exhibited the lowest calculated encapsidation efficiency (approximately 40% of the control). This result reflects the fact that the amount of RV-1 reached only the median value in the nuclease-resistant (encapsidated) pool of DNA; whereas its proportion in the total extrachromosomal DNA sample was above the mean of vector amounts (Fig. 3A). RV-2 that positively scored in the REPs assays was over-represented in both DNA samples (Fig. 3B), but the nuclease-resistant-to-total DNA proportion was similar to the control vector. Altogether, the results shown in Fig. 3 suggest that the RV packaging in this experimental system is not enhanced by any distinct MPyV element and that the second MPyV ori region in RV-1 can even negatively influence encapsidation. This result also suggests that the presence of the SV40 ori and *ses* in all RVs may drive the replication and/or packaging, overcome the putative MPyV encapsidation signaling and limit the usage of this experimental design. To dissect the role of these elements in vector packaging, we performed additional experiments in a mouse production cell line.

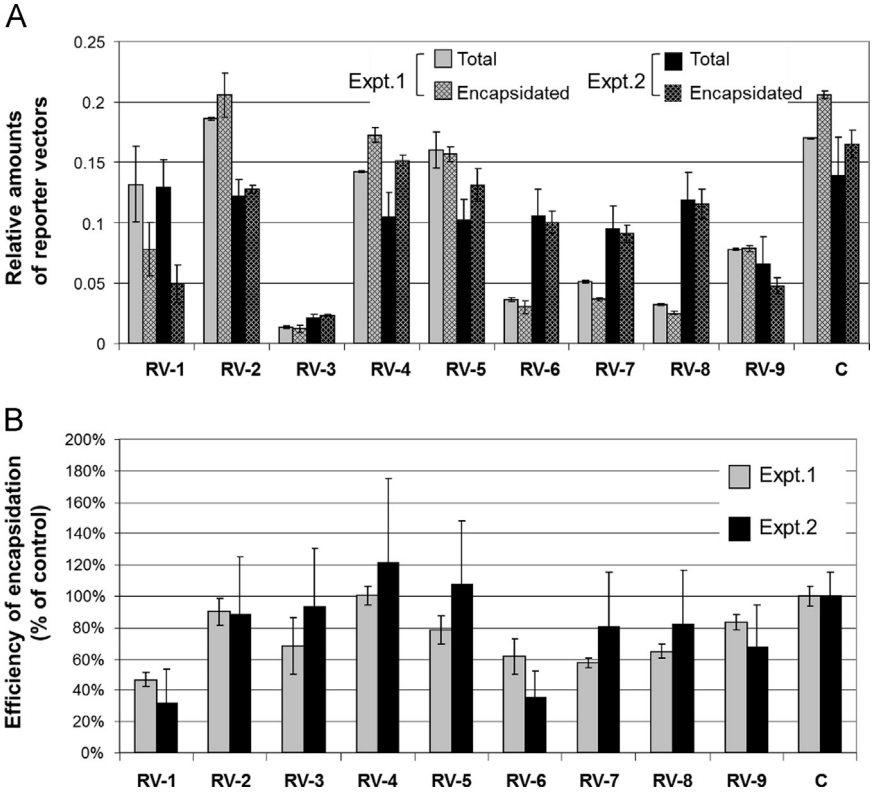

Fig. 3. The determination of reporter vector encapsidation efficiency by qPCR. All 10 RVs (including pGL3-Control) were mixed together with helper vectors ph2-VP1 and ph3β and were transfected into the 293TT cell line. Cells were harvested at 48 h post-transfection. The total extrachromosomal DNA and nuclease-resistant DNA were extracted separately from equivalent numbers of cells. Both DNA samples were used for qPCR with primers that were specific for each RV to determine the amount of each RV. The data were normalized to the luciferase gene to calculate the relative amount of each RV in both DNA pools. (A) The relative amounts of each RV in the extrachromosomal (Total) and nuclease-resistant (Encapsidated) DNA samples are shown for two independent experiments as the means of triplicate samples ± standard deviations (error bars). (B) The encapsidation efficiency was determined from the data that are shown in (A) as a ratio between the relative amounts of each RV that were detected in the nuclease-resistant pool and in the total extrachromosomal DNA. The data are plotted as percentages of encapsidation efficiencies compared to the control (C) RV (set as 100%). Error bars represent the standard deviations of triplicate samples.

Reporter vectors can be encapsidated into MPyV pseudovirions without replication

To separate the driving force of SV40-based replication and the possible influence of *ses* on encapsidation, we performed additional experiments in WOP cells. The WOP cell line is a mouse cell line expressing MPyV T-antigens, which support the replication of plasmids containing the MPyV ori and therefore only RV-1 out of all of the RVs is able to replicate in these cells. Originally, we attempted to produce MPyV pseudovirions using the same reporter and helper vectors as in the REPs assay. However, when the expression of the capsid and reporter genes after co-transfection was assessed using immunofluorescence, we discovered that the co-transfection/expression rate was low in these particular cells (less than 10% of transfected cells expressed luciferase together with all three capsid proteins; data not shown). For this reason, further experiments were performed with the pCG-VP1/2/3 vector, which ensured the weak, but simultaneous, expression of all three capsid genes in one cell. Moreover, the mouse producer cells allowed us to use the MPyV genomic DNA as the helper vector for the production of high levels of all capsid proteins. Pseudovirions generated in WOP cells were further analyzed by transformation assay: the encapsidated plasmids that were extracted as nuclease-resistant DNA were used for the transformation of *Escherichia coli*. To reveal their identity, the RVs were isolated from ampicillin-resistant colonies and then sequenced.

In the initial experiment, we used an equimolar mix of all RVs (including the pGL3-Control) as the target DNA to determine whether any RVs would show preferential encapsidation. In the second set of experiments, only the pGFPmax vector served as the target DNA. The vector pGFPmax does not contain any sequences that were derived from either MPyV or the SV40 genome and has a suitable size (3.8 kb) for encapsidation. Using this target vector, we wanted to determine whether a non-replicating vector without *ses* and MPyV sequences could be successfully encapsidated. Target vectors were used for transfections with either MPyV DNA or pCG-VP1/2/3 as the helper vector (Table 2). Control transfections without the helper vector were also performed (see Table 2). Cells were harvested at 48 h post-transfection, and total extrachromosomal and nuclease-resistant DNAs were extracted from an equivalent number of cells and were used for qPCR quantification and *E. coli* transformation, respectively. The overall results of this assay, which were expressed as numbers of *E. coli* colonies grown after transformation with each sample, are shown in Table 2. The transformation of DNA that was extracted from pseudovirions formed with the use of MPyV DNA as the helper vector led to the emergence of 26 colonies; whereas the pCG-VP1/2/3 vector co-transfection samples yielded 166 colonies. Notably, in contrast to pCG-VP1/2/3, MPyV DNA could compete with the encapsidation of RVs, and the low colony yield was expected. The control reaction without the transfected helper vector yielded no colonies, indicating that the nuclease treatment was effective. Subsequently, plasmid DNA from 52 colonies (all colonies from the MPyV DNA sample and 26 randomly chosen colonies grown from the pCG-VP1/2/3 sample) was extracted and sequenced. In parallel, the total extrachromosomal DNA that was extracted from the same samples of transfected cells was subjected to qPCR to determine the total amount of each individual RV. The frequency of each RV that was found in the nuclease-resistant pool and its proportion relative to the total amount of each vector in the extrachromosomal DNA are shown in Table 3. The data from the sample in which MPyV DNA served as a helper indicate that the high total amount of RV-2 in the transfected cells led to its preferential encapsidation. In contrast, the amplification of RV-1 was likely negatively influenced by the presence of the replicating viral genome, thus decreasing the probability of its encapsidation. However, sequence analysis of 26 clones from randomly chosen colonies that were obtained from the sample in which the pCG-VP1/2/3 vector served as a helper revealed that RV-1 had been encapsidated with the highest frequency. The high total amount of RV-1 in the extrachromosomal DNA fraction also indicated that its replication was not influenced by the pCG-VP1/2/3 helper DNA (as observed for MPyV helper DNA; see Table 3). Although it is impossible to determine the encapsidation efficiency for each vector because of the low number of sequenced clones, the data suggest that encapsidation in the mouse production system does not exhibit a strict sequence preference and reflects the RV amount. Spearman's rank correlation test (Wessa, 2012) confirmed a very strong positive correlation [$\rho(8)=0.852$, $p=0.001$] between the amount and frequency of encapsidation of a given RV in the MPyV DNA helper sample and a strong positive correlation [$\rho(8)=0.705$, $p=0.023$] in the pCG-VP1/2/3 helper sample, which was statistically significant. Moreover, a second set of experiments with the pGFPmax vector as a target DNA (Table 2) was performed to reveal whether the SV40 encapsidation signal is dispensable for packaging. The numbers of colonies that are shown in Table 2 indicate that the pGFPmax vector, which lacks SV40 elements, could be efficiently encapsidated into the viral particles. Taken together, these results suggest that the vector amount is the only important factor in encapsidation and that replication competence, which is connected with the presence of SV40 and MPyV regulatory elements, is not necessary for encapsidation.

Table 2
Summary of transfections performed in WOP cells for the extraction of nuclease-resistant DNA used for *E. coli* transformation.

Vector		Transfection mix					
Helper	MPyV DNA	+	−	−	+	−	−
	pCG-VP1/2/3	−	+	−	−	+	−
Target	RVs mix[a]	+	+	+	−	−	−
	pGFP max	−	−	−	+	+	+
No. of colonies	Expt. 1	**26**[b]	**166**[c]	**0**	**81**	**146**	**0**
	Expt. 2	**ND**	**ND**	**ND**	**65**	**67**	**0**

[a] Equimolar ratios of all RVs were used in the transfection.
[b] All colonies were used for sequencing.
[c] 26 colonies were randomly chosen for sequencing; ND – not done.

Table 3
Encapsidation as determined from transformation assays.

	Helper: MPyV DNA		Helper: pCG-VP1/2/3	
	No. of colonies[a]	Relative amount[b]	No. of colonies[a]	Relative amount[b]
RV-1	0	0.10	19	0.25
RV-2	11	0.23	2	0.17
RV-3	0	0.02	0	0.01
RV-4	1	0.11	0	0.10
RV-5	4	0.13	1	0.11
RV-6	0	0.06	2	0.09
RV-7	3	0.07	0	0.08
RV-8	0	0.03	0	0.03
RV-9	3	0.10	1	0.05
Control	4	0.15	1	0.10
Total	26	1	26	1

[a] Number of colonies that contain a given RV.
[b] The relative amount of each RV in the sample was determined by qPCR.

Some regions of the MPyV genome enhance the stability of DNA after transfection

Finally, we focused on RV-2 and its unique qualities that led to its positive scoring in the REPs assay (Fig. 2) and partly in the

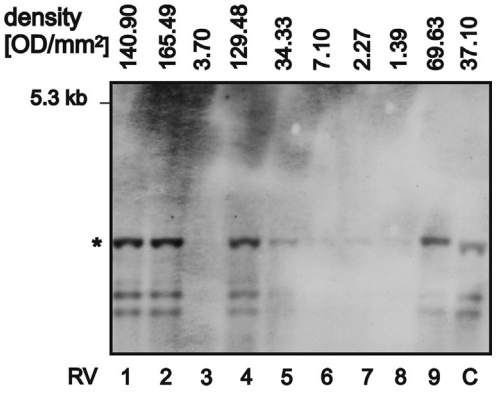

Fig. 4. Determination of the intracellular amounts of reporter vectors. 3T3 cells were transfected with individual RVs. The total extrachromosomal DNA was extracted at 48 h post-transfection and was digested with Sal I and Dpn I enzymes. DNA from each sample (0.5 μg) was separated using agarose electrophoresis, subjected to Southern blotting, hybridized to a DIG-labeled luciferase gene probe, detected with chemiluminescence and recorded by exposure to X-ray film. The optical density of the prominent band (indicated by an asterisk) was determined with a densitometer and is shown in the upper row for each sample.

transformation assay (Table 2). We repeatedly observed that, compared with other RVs, the direct transfection of RV-2 into cells leads to very high luciferase activity in all cell lines tested (NIH-3T3, 3T6, COS-1, 293TT and WOP cells, data not shown). Our results from qPCR (Fig. 3B) also suggested that this effect was more likely caused by an augmented amount of the vector than by the transcriptional upregulation of the activity of the reporter gene in transfected cells. To examine whether the early intron region (nucleotides 292–985) of the MPyV genome can confer some unusual property on RV-2 that leads to the stabilization of the plasmid (due to the plasmid maintenance sequences) or to ori-independent replication, we transfected individual RVs into the mouse NIH-3T3 cell line and followed the reporter activity in several generations of cells. The NIH-3T3 cell line is a parental line for WOP cells and does not normally support the replication of MPyV ori-containing plasmids without the presence of large T-antigen. After the transfection of a full set of RVs into this cell line, RV-2 exhibited the highest luciferase activity, even after three cell passages (data not shown). We performed a replication assay to exclude the possibility that an ori-independent, low level of plasmid replication, which is occasionally observed (Katinka and Yaniv, 1983), is responsible for this effect. The total DNA from cells that were transfected by nucleofection was extracted, digested with Dpn I and analyzed by gel electrophoresis and Southern blotting. Dpn I is a methylation-sensitive enzyme; newly replicated DNA molecules are resistant to Dpn I digestion. The results showed that none of the RVs could be replicated in 3T3 cells (Fig. 4; note complete digestion by Dpn I). More importantly, a great difference in the abundance of individual plasmids was noted, although the transfection was performed with equal amounts of RVs. Because the Amaxa Nucleofector Technology is considered the most reproducible method of transfection, the results suggest that RV-2, RV-1 and RV-4 harbor MPyV elements that are responsible for the increased intracellular stability of DNA after transfection. RV-2 was the most stable (Fig. 4).

Discussion

In this study, we attempted to identify the MPyV encapsidation signal. We used pseudovirion-based approaches for the identification of the polyomavirus-specific sequences that enhance the encapsidation of RVs.

From the series of assays, only the most laborious, transformation assay led to the final conclusion that the encapsidation of target DNA is not driven by any distinct, sequence-specific encapsidation signal. The assay confirmed a strong positive correlation between the amount and frequency of encapsidation of a given RV. Moreover, we used two different helpers (pCG-VP1/2/3 or MPyV DNA) for the production of pseudovirions in WOP cells and both variants brought additional interesting results. The presence of the MPyV DNA as a replicating and transcriptionally active viral genome creates a highly competitive environment in the system. This environment probably negatively affected RV-1 replication because its relative intracellular amount was lower than expected for a vector with an exclusive capacity to replicate. It is well-documented that the presence of wild-type replicons strongly interferes with mutant replication after transfection (Nilsson et al., 1991) and the defect could be at the stage of initiation (Yamaguchi and DePamphilis, 1986). Furthermore, MPyV DNA is undoubtedly efficiently encapsidated into virions but cannot be detected by this type of assay. Therefore, the RVs that were identified with a frequency that was related to their intracellular amounts reflect the minority of successfully encapsulated DNA. In contrast, the encapsidation of pCG-VP1/2/3 is prevented due to a size discrimination mechanism, and the vector did not restrict the replication potential of RV-1, which accumulated in the system. Subsequently, the high relative amount of RV-1 led to its preferential encapsidation. However, in both cases, the non-replicating pGFPmax vector without any viral (MPyV or SV40) sequence could be encapsidated equally well. The results indicated that an intracellular amount of target DNA determines the outcome of the encapsidation process, and virtually any DNA with an appropriate size can be encapsidated. The preferential packaging of a RV with an active regulatory region can be a consequence of the fact that encapsidation is likely interconnected with the processes of replication and/or transcription. According to our observations, at the early stages of the late phase of MPyV infection, the newly expressed VP1 protein accumulates at distinct sites of DNA replication near PML bodies; whereas during the later times, the VP1 protein expands throughout the nucleus (Ryabchenko, unpublished data). Therefore, the organization of the replication foci during the initial phases of virion formation, when capsid proteins are spare, can favor DNA with a highly active ori region for encapsidation. In agreement with this notion, in our mouse production system, where the pCG-VP1/2/3 vector ensures a low level of capsid proteins (thus reminiscent of early events in the late phase of infection), RV-1 containing an active ori was detected as an almost exclusive target of encapsidation (Table 3). In contrast, MPyV helper DNA played a dual role in the mouse system by encapsidating itself as replicating target DNA in the initial phase of virion formation and ensuring high levels of capsid proteins later, thus allowing the encapsidation of other available targets. We propose that by employing such a simple regulation of encapsidation that is based on the spatio-temporal organization of virion formation, MPyV can achieve a reasonable level of encapsidation specificity without employing a sequence-specific signal.

The results from the qPCR assay were also in agreement with this notion. None of the MPyV sequences were able to enhance the encapsidation of RVs and the RV-1 with second MPyV ori region was packaged with the lowest efficiency. It has been shown that SV40 LT can efficiently bind the MPyV ori on LT-binding sites (Bhattacharyya et al., 1995) without triggering DNA replication. We reasoned that SV40 LT binding on a non-active MPyV ori could cause the retention of target DNA on chromatin and limit its encapsidation.

The REPs system, originally designed as an easy and rapid screening method, was unfortunately biased as a result of unexpected obstacles. The idea of the REPs system is based on the

assumption that intracellular levels of each RV in producer cells are comparable due to the similar efficiency of replication driven from a common SV40 origin. Normalizing the transfection efficiency of the system by the co-transfected internal control gene (for β-galactosidase) and by the protein content in the lysate of the producer cell should provide adequate corrections for perturbations during the transduction assay. An analysis of the REPs screening hit (RV-2) revealed that this is not the case. The experiments that took into consideration the levels of copies of individual RVs showed a constant and high level of RV-2 in cells, and consequently, in viral particles in most assays (see Fig. 3B; Table 3). By performing the replication assay with RVs that were transfected into 3T3 cells, we excluded the possibility that the plasmids undergo an ori-independent replication; however, without further analyzing the reasons, we observed completely different intracellular amounts of each RV after transfection. It has previously been shown that the use of a second reporter plasmid as an internal standard to normalize luciferase activity in transient transfection experiments may lead to a systematic error due to the interaction between co-transfected RVs (Huszár et al., 2001, Bergeron et al., 1995). Moreover, it has been shown that coexpression efficiencies among transfected cells are variable, and a significant number of transfected cells express only a single target protein (Ma et al., 2007). This observation points to the difficulties that are connected with the comparison of the quantitative data from parallel transfections in general; however, in our system, the small error in the normalization of the cell lysates of producer cells can be dramatically amplified during the final screen in the detection cell line where the RV replicates. We concluded that this amplification was the case for RV-2 in the REPs assay.

Until now, the SV40 *ses* sequence has been the only identified encapsidation signal in polyomaviruses. Our work surprisingly suggests the fact that the mechanism of the selection of DNA for encapsidation may not be universal within the virus family. We hypothesize that the mechanisms of encapsidation in MPyV and SV40 may likely differ due to the differences in the DNA-binding properties of the minor capsid proteins. The minor proteins of SV40 bind nonspecifically to DNA (Dean et al., 1995), and Sp1 proteins cooperate in *ses*-specific DNA binding with VP2/3 (Gordon-Shaag et al., 2002); whereas MPyV VP2 and VP3 proteins do not bind the DNA (Chang et al., 1993) None of these proteins interact with the MPyV genome in virions (Carbone et al., 2004). However, it should be noted that the SV40 mutant that lacked minor structural proteins is able to encapsidate virus DNA efficiently (Nakanishi et al., 2007), which is similar to the corresponding MPyV mutants (Mannova et al., 2002). Moreover, in vitro experiments have shown that the *ses* sequence itself, in the absence of a large excess of cellular chromatin, does not promote the preferential binding of capsomere VP1(5)VP2/3 complexes to the SV40 genome (Roitman-Shemer et al., 2007). These observations not only suggest that the involvement of these proteins in the encapsidation process may differ between both viruses but also that their importance may depend on the actual context. Further investigation would be needed to evaluate the preferential mode of genome selection during virion assembly for other members of the *Polyomaviridae* family.

Materials and methods

Plasmids and viruses

For the construction of RVs, the plasmid pGL3-Control (Promega, Madison, Wisconsin, USA) was shortened by digestion with the restriction enzyme Ssp I and re-ligation (without the f1 ori sequence). The obtained pGL3-ΔC vector could accommodate polyomaviral sequences without exceeding the size of the MPyV genome. Both vectors, pGL3-Control and pGL3-ΔC, yielded the same level of activity of the reporter luciferase gene that they carried. The genome of the MPyV BG strain (GenBank: AF442959.1) was divided into nine regions of approximately 650 bp each, and each region was cloned using the In-Fusion HD Cloning System (Clontech Laboratories, Mountain View, California, USA) into the Mlu I and Xho I sites of the pGL3-ΔC plasmid. All clones were verified by sequencing. A list of the cloning primers used is available as Supplementary information in Table S1.

The helper vector ph2-VP1 (7802 bp), which carries the codon-modified VP1 and VP2 MPyV genes as described in Tolstov et al. (2009), was constructed from the ph2p vector (Addgene plasmid 22520) (Pastrana et al., 2009) by digestion with the restriction enzymes Stu I and Cla I and re-ligation with the PCR-amplified product, which contains the human elongation factor-1 alpha (hEF1a) promoter, codon-modified VP1 gene, woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) sequence and hEF1a poly(A) signal from the pwP vector (Addgene plasmid 22519) (Pastrana et al., 2009). The vector ph3β (7538 bp), which carries the codon-modified MPyV VP3 and β-galactosidase genes, was constructed from ph3p (Addgene plasmid 22521) (Pastrana et al., 2009) by digestion with the restriction enzymes Stu I and Not I and re-ligation with the β-galactosidase gene, which was obtained via Sma I and Not I digestion of the pCMVβ vector (Clontech Laboratories, Mountain View, California, USA). The vector pCG-VP1/2/3 (6542 bp) was constructed from the pGL3-Control vector by the excision of the luciferase gene using Ehe I and Xba I restriction enzymes and re-ligation with a part of the polyomavirus genome that is composed of the regulatory and late-gene regions (cut by Ehe I and Xba I from MPyV genome DNA). The plasmid pmaxGFP (Lonza, Cologne, Germany), which encodes maxGFP, which is a green fluorescent protein from the copepod *Pontellina plumata*, was used as a negative control vector in the transfections and as a target DNA for some packaging experiments. The plasmid pcDNA3-LT was used for the expression of the MPyV LT antigen in some co-transfection experiments. The summarized description of all plasmids that were used for the production of the pseudovirion is shown in supplementary Table S2.

Cell lines and transfections

Mouse Swiss albino 3T6, NIH 3T3 mouse fibroblasts, SV40-transformed African green monkey kidney cells, COS-1 and WOP cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich, St. Louis, Missouri, USA) that was supplemented with 10% Gibco™ fetal calf serum (Invitrogen, Paisley, UK) and GlutaMAX™ (Invitrogen, Paisley, UK) at 37 °C in a 5% CO_2-air humidified incubator. The mouse WOP cell line that constitutively expresses the MPyV large T-antigen is a 3T3 fibroblast derivative that was transformed using an origin-defective polyomavirus (Dailey and Basilico, 1985). The human embryonic kidney cells 293TT that express the SV40 large T-antigen were kindly provided by John Schiller and Chris Buck (Bethesda, Maryland, USA) and were cultivated as previously described (Buck and Thompson, 2007).

The plasmid DNA that was used for transfections was purified using either the EndoFree Plasmid Maxi Kit (Qiagen, Valencia, California, USA) or the GenBond Plasmid Endofree FlexSpin Kit (Renogen Biolab, Vancouver, Canada). 293TT or COS-1 cells were transfected using the TurboFect Transfection Reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturers' instructions. Briefly, logarithmically growing cells (5×10^5) in 6-well dishes were transfected with 4 μg of DNA. In the REPs assay, 1.5 μg of ph2-VP1 and 0.5 μg of ph3β together with 2 μg of RV were transfected in a production cell line. WOP cells

(for transformation assays) and 3T3 cells (for the replication assay) (both 4×10^6) were transfected (using Amaxa® Nucleofector® Technology [Lonza, Cologne, Germany] according to the manufacturer's instructions) with 6 μg of DNA (total) in solution V using the programs U-030 and T-030, respectively. Specifically, the mixed transfections (all RVs with helper DNA) contained either 2.5 μg of ph2VP1 and 0.5 μg of ph3β or 3 μg of pCG-VP1/2/3 as helper DNA and 0.3 μg of each RV. For the comparative analysis of the encapsidation of the pGFPmax vector, either 3 μg of MPyV DNA or 3 μg of pCG-VP1/2/3 were mixed with 3 μg of the pGFPmax plasmid and were transfected into WOP cells.

Pseudovirion production and purification

Transfected 293TT cells were harvested 48 h post-transfection, and pseudovirions were isolated by three rounds of freeze–thaw cycles as described for polyomavirus (Türler and Beard, 1985), concentrated by pelleting through a 10% sucrose cushion (25,000 r.p.m., Beckman SW28 rotor, 3 h, 4 °C), resuspended in B buffer (150 mM NaCl, 10 mM Tris–HCl [pH 7.4], 0.01 mM $CaCl_2$) and purified by CsCl gradient ultracentrifugation (Beckman SW41 rotor, 35,000 r.p.m., 24 h, 18 °C). Gradient fractions were collected by bottom puncture and were assayed for the presence of the VP1 protein by dot-blot analysis. The fractions that contained a peak amount of VP1 (1.33–1.29 g/cm^3) were dialyzed against B buffer, concentrated by pelleting through a 10% sucrose cushion (Beckman SW41 rotor, 35,000 r.p.m., 2 h, 4 °C) and subjected to electron microscopic examination.

REPs (transduction) assay

Producer 293TT cells (1.5×10^6) were harvested at 48 h post-transfection, washed by PBS and lysed in 100 μl of 250 mM Tris–HCl [pH 7.4] by three rounds of freeze–thaw cycles. Lysates were centrifuged at 10,000*g* for 5 min at 4 °C. The supernatant (50 μl) was supplemented with magnesium chloride (final concentration 10 mM), treated with 0.1 mg/ml DNase I (Roche Diagnostics GmbH, Mannheim, Germany) for 30 min in 25 °C and used for transduction. An aliquot of the original supernatant (20 μl) was used for the parallel determination of the activity of luciferase and β-galactosidase with the Dual-Light System (Applied Biosystems, Bedford, Massachusetts, USA) and for the measurement of the protein concentration (Bradford, 1976). Several cell lines were tested as suitable detection cell lines; COS-1 cells were chosen for the final transduction screen. Exponentially growing COS-1 cells in a 6-well dish (1.5×10^5 cells/well) were washed with 2 ml of serum-free medium, and 50 μl of the lysate supernatant was diluted in 150 μl of serum-free medium, added to cells and incubated for 1.5 h in a thermostat with intermittent agitation. At the end of the incubation, 2.5 ml of complete medium was added to the cells. Transduced COS-1 cells were harvested at 48 h post-transduction and were washed two times with phosphate-buffered saline (PBS). The cell lysate was prepared by three rounds of freeze–thaw cycles, and cellular debris was removed by centrifugation at 14,000*g* and 4 °C for 5 min. Supernatants were used for protein concentration (Bradford, 1976) and luciferase measurement determinations with the Luciferase Assay System (Promega, Madison, Wisconsin, USA) in a Microlite TLX2 luminometer (Dynatech Laboratories, Inc, Chantilly, Virginia, USA). The transduction efficacy, which was expressed as relative luciferase activity (in relative luminescence units) for each sample, was calculated by dividing the relative activity of luciferase that was measured in COS-1 cell lysates by the relative activity of luciferase that was measured in the 2932TT cell lysate. The luciferase activities that were measured in the 2932TT cell lysate were normalized by protein concentrations and β-galactosidase activity (i.e., the relative activity of luciferase in 293TT cells), and the luciferase activities that were measured in COS-1 cells were normalized by protein concentrations only (i.e., the relative activity of luciferase in COS-1 cells). The background activity of luciferase that was measured in the control sample that was transduced with lysates, which were generated from 293TT cells that were transfected with pGL3-Control vector without helper vectors, was subtracted from all measurements before computation.

DNA analysis

Low-molecular-weight (extrachromosomal) DNA was extracted from one million cells using a previously reported neutral lysis method (Arad, 1998). The extraction of nuclease-resistant extrachromosomal DNA from transfected cells was performed exactly as described in (Buck et al., 2004). The viral genomic DNA that was used as helper in transfection assays was extracted according to the classical Hirt procedure (Hirt, 1967). For Southern blot analysis, the purification of total DNA from transfected cells was performed using a DNeasy Blood & Tissue Kit (Qiagen, Valencia, California, USA).

Transformation assay

WOP cells that were transfected with mixed vectors (all RVs and helper DNA) were harvested at 48 h post-transfection; aliquots were then generated by dividing each lysate in half. The extrachromosomal and nuclease-resistant (encapsidated) DNAs were extracted from each aliquot as described above. The nuclease-resistant DNA was used to transform Stellar™ Competent Cells (*E. coli* HST08 strain) (Clontech Laboratories, Mountain View, California, USA). The plasmid DNA from transformed bacterial colonies was extracted using a QIAGEN Plasmid Mini Kit (Qiagen, Valencia, California, USA) and was sequenced with a 3130xl Genetic Analyzer (Applied Biosystems, Bedford, Massachusetts, USA). Extrachromosomal DNA was used for quantitative polymerase chain reaction (qPCR) analysis to determine the total intracellular amounts of each individual RV in WOP cells. For the analysis of the encapsidation of the pGFPmax vector, Stellar™ Competent Cells were transformed with the nuclease-resistant DNAs. Equal amounts of the bacterial suspension were plated on agar plates containing kanamycin and ampicillin to discriminate between pGFPmax (kanamycin resistance) and the helper vector (ampicillin resistance).

Electron microscopy

For the ultrastructural analysis of cell-associated viral particles, the producer cells were washed with PBS at 48 h post-transfection and were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer on ice for 60 min. Cells were washed two times with cacodylate buffer and were postfixed with 1% osmium tetroxide for 60 min. The postfixation was followed by embedding the cells in 3% low melting point agarose. The solidified agarose blocks were cut into 1 mm^3 pieces and were dehydrated with an increasing ethanol series (30%, 50%, 70%, 90%, 96%, 100% and 100%), each for 15 min. Dehydrated blocks were infiltrated with an increasing series of AGAR 100 resin (Gröpl, Tulln, Austria) in propylene oxide (propylene oxide: AGAR 100-2 × pure propylene oxide 10 min); 2:1 (15 min); 1:1 (30 min); 2:1 (30 min); and pure AGAR 100 overnight; pure AGAR 100 (3 h). Polymerization was performed at 60 °C for 72 h. Sections of 70-nm thickness (Leica ultramicrotome EM UC7, Leica Microsystems, Austria) were contrasted with a saturated water solution of uranyl acetate (5 min) and Reynolds lead citrate solution (3 min).

For the negative staining of purified viral particles, the parlodion-carbon coated grids, which were activated by glow discharge, were put

on the top of a 10 μl-drop of sample and were allowed to adsorb for 5 min; the grids were then rinsed in 2 drops of filtered distilled water and transferred onto 2 drops of 2% phosphotungstic acid (pH 7.3), left for 1 min and then dried.

Electron micrographs were recorded in a JEM-1011 electron microscope (JEOL) operating at 80 kV.

Quantitative PCR

qPCR was performed in a Light Cycler 480 II (Roche Diagnostics GmbH, Mannheim, Germany) using the iQ™ SYBR® Green Supermix (Bio Rad), according to the manufacturer's protocol. The DNA was amplified by PCR using a forward primer from Promega (RVprimer_pGL3), which was common to all RV and reverse primers that were designed for each individual RV as described in the supplementary Table S3. Specific primer pairs were designed for pGL3-Control and the luciferase gene. The quantification of each RV in the extracted DNA samples was performed with the Light Cycler 480 II software for an advanced relative quantification with an efficiency correction using standard curves for each primer pair. The concentration of each RV was normalized to the luciferase gene and was expressed as a proportion of the individual vector in the total amount of DNA.

Replication assay and Southern blotting

The 3T3 cells that were transfected with individual vectors by nucleofection were harvested at 48 h post-transfection for the extraction of total DNA. The DNA from each sample (0.5 μg) was digested with Sal I and Dpn I enzymes, separated by electrophoresis in 0.8% agarose and blotted onto a nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany). Southern blotting was performed with a DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. The hybridization was performed using a DIG-labeled probe that contained the luciferase gene probe. The blots were detected with chemiluminescence and were recorded by exposure to X-ray film. The optical densities of the bands were determined with a GS-800™ Calibrated Densitometer (Bio-Rad Laboratories, Hercules, California, USA).

Immunofluorescence

The cells that were grown on the glass slides were fixed in 3.7% paraformaldehyde (PFA) in PBS for 15 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min, rinsed 3 times with PBS and blocked with PBS containing 0.25% bovine serum albumin and 0.25% porcine skin gelatin for 30 min. Immunostaining with primary and secondary antibodies was performed for 1 h and 30 min, respectively, with extensive washing by PBS after each incubation step. The cells were mounted in 50% glycerol with 4′,6-diamidino-2-phenylindole (DAPI) and were examined using an Olympus BX-60 fluorescence microscope (Olympus; Center Valley, Pennsylvania, USA).

Antibodies

The following primary antibodies were used: rat monoclonal IgG against the MPyV large T-antigen (LT1) (Dilworth and Griffin, 1982), mouse monoclonal anti-MPyV VP1 IgG, mouse monoclonal IgG against the common region of VP2 and VP3 (Forstová et al., 1993) and the goat anti-luciferase polyclonal antibody (pAb) (Promega, Madison, Wisconsin, USA). The following secondary antibodies were used: donkey anti-mouse IgG and donkey anti-rat IgG conjugated with Alexa Fluor 488, goat anti-rat and donkey anti-goat IgGs conjugated with Alexa Fluor 546 and chicken anti-mouse IgG conjugated with Alexa Fluor 647 (all from Molecular Probes®, Invitrogen, Paisley, UK).

Acknowledgments

This work was generously supported by the Grant Agency of Czech Republic GACR, No. P302-10-P118 (H.S.), by SVV-2013-267205 (J.S., J.S.), by TACR No. 213TA0310700 (M.F.), by a project of the Ministry of Education, Youth and Sports of the Czech Republic, MSM0021620858 (J.F.), and by UNCE 204013/2013.

We thank Vlasta Sakařová for excellent technical assistance.

Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.12.010.

References

Arad, U., 1998. Modified Hirt procedure for rapid purification of extrachromosomal DNA from mammalian cells. Biotechniques 24, 761–762.
Bergeron, D., Barbeau, B., Léger, C., Rassart, E., 1995. Experimental bias in the evaluation of the cellular transient expression in DNA co-transfection experiments. Cell. Mol. Biol. Res. 41, 155–159.
Bhattacharyya, S., Lorimer, H.E., Prives, C., 1995. Murine polyomavirus and simian virus 40 large T antigens produce different structural alterations in viral origin DNA. J. Virol. 69, 7579–7585.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Buck, C.B., Pastrana, D.V., Lowy, D.R., Schiller, J.T., 2004. Efficient intracellular assembly of papillomaviral vectors. J. Virol. 78, 751–757.
Buck, C.B., Thompson, C.D., 2007. Production of papillomavirus-based gene transfer vectors. Curr. Protoc. Cell. Biol. 37, 26.1.1–26.1.19.
Carbone, M., Ascione, G., Chichiarelli, S., Garcia, M.I., Eufemi, M., Amati, P., 2004. Chromosome-protein interactions in polyomavirus virions. J. Virol. 78, 513–519.
Chang, D., Cai, X., Consigli, R.A., 1993. Characterization of the DNA binding properties of polyomavirus capsid protein. J. Virol. 67, 6327–6331.
Chen, X.J.S., Stehle, T., Harrison, S.C., 1998. Interaction of polyomavirus internal protein VP2 with the major capsid protein VP1 and implications for participation of VP2 in viral entry. EMBO J. 17, 3233–3240.
Dailey, L., Basilico, C., 1985. Sequences in the polyomavirus DNA regulatory region involved in viral DNA replication and early gene expression. J. Virol. 54, 739–749.
Dalyotherman, N., Bennunshaul, O., Gordonshaag, A., Oppenheim, A., 1996. The simian virus 40 packaging signal ses is composed of redundant DNA elements which are partly interchangeable. J. Mol. Biol. 259, 69–80.
Dean, D.A., Li, P.P., Lee, L.M., Kasamatsu, H., 1995. Essential role of the Vp2 and Vp3 DNA-binding domain in simian virus 40 morphogenesis. J. Virol. 69, 1115–1121.
Dilworth, S.M., Griffin, B.E., 1982. Monoclonal antibodies against polyoma virus tumor antigens. Proc. Natl. Acad. Sci. USA 79, 1059–1063.
Forstová, J., Krauzewicz, N., Wallace, S., Street, A.J., Dilworth, S.M., Beard, S., Griffin, B.E., 1993. Cooperation of structural proteins during late events in the life cycle of polyomavirus. J. Virol. 67, 1405–1413.
Gendron, D., Delbecchi, L., Bourgauxramoisy, D., Bourgaux, P., 1996. An enhancer of recombination in polyomavirus DNA. J. Virol. 70, 4748–4760.
Gordon-Shaag, A., Ben-Nun-Shaul, O., Kasamatsu, H., Oppenheim, A.B., Oppenheim, A., 1998. The SV40 capsid protein VP3 cooperates with the cellular transcription factor Sp1 in DNA-binding and in regulating viral promoter activity. J. Mol. Biol. 275, 187–195.
Gordon-Shaag, A., Ben-Nun-Shaul, O., Roitman, V., Yosef, Y., Oppenheim, A., 2002. Cellular transcription factor Sp1 recruits simian virus 40 capsid proteins to the viral packaging signal, ses. J. Virol. 76, 5915–5924.
Guo, Z.S., Depamphilis, M.L., 1992. Specific transcription factors stimulate simian virus 40 and polyomavirus origins of DNA replication. Mol. Cell. Biol. 12, 2514–2524.
Hirt, B., 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. 26, 365–369.
Huszár, T., Mucsi, I., Terebessy, T., Masszi, A., Adamkó, S., Jeney, C., Rosivall, L., 2001. The use of a second reporter plasmid as an internal standard to normalize luciferase activity in transient transfection experiments may lead to a systematic error. J. Biotechnol. 88, 251–258.
Katinka, M., Yaniv, M., 1983. DNA replication origin of polyoma virus: early proximal boundary. J. Virol. 47, 244–248.
Ma, Z.L., Werner, M., Körber, C., Joshi, I., Hamad, M., Wahle, P., Hollmann, M., 2007. Quantitative analysis of cotransfection efficiencies in studies of ionotropic glutamate receptor complexes. J. Neurosci. Res. 85, 99–115.

Mannova, P., Liebl, D., Krauzewicz, N., Fejtova, A., Stokrova, J., Palkova, Z., Griffin, B. E., Forstova, J., 2002. Analysis of mouse polyomavirus mutants with lesions in the minor capsid proteins. J. Gen. Virol. 83, 2309–2319.

Martelli, F., Iacobini, C., Caruso, M., Felsani, A., 1996. Characterization of two novel YY1 binding sites in the polyomavirus late promoter. J. Virol. 70, 1433–1438.

Nakanishi, A., Itoh, N., Li, P.P., Handa, H., Liddington, R.C., Kasamatsu, H., 2007. Minor capsid proteins of simian virus 40 are dispensable for nucleocapsid assembly and cell entry but are required for nuclear entry of the viral genome. J. Virol. 81, 3778–3785.

Nilsson, M., Osterlund, M., Magnusson, G., 1991. Analysis of polyomavirus enhancer-effect on DNA replication and early gene expression. J. Mol. Biol. 218, 479–483.

Oppenheim, A., Peleg, A., 1989. Helpers for efficient encapsidation of SV40 pseudovirions. Gene 77, 79–86.

Oppenheim, A., Sandalon, Z., Peleg, A., Shaul, O., Nicolis, S., Ottolenghi, S., 1992. A cis-acting DNA signal for encapsidation of simian virus 40. J. Virol. 66, 5320–5328.

Palkova, Z., Spanielova, H., Gottifredi, V., Hollanderova, D., Forstova, J., Amati, P., 2000. The polyomavirus major capsid protein VP1 interacts with the nuclear matrix regulatory protein YY1. FEBS Lett. 467, 359–364.

Pastrana, D.V., Tolstov, Y.L., Becker, J.C., Moore, P.S., Chang, Y., Buck, C.B., 2009. Quantitation of Human Seroresponsiveness to Merkel Cell Polyomavirus. PLoS Pathog., 5.

Roitman-Shemer, V., Stokrova, J., Forstova, J., Oppenheim, A., 2007. Assemblages of simian virus 40 capsid proteins and viral DNA visualized by electron microscopy. Biochem. Biophys. Res. Commun. 353, 424–430.

Spanielová, H., 2002. Analysis of regulation of polyomavirus promoters by a luciferase reporter system. Acta Virol. 46, 219–227.

Tolstov, Y.L., Pastrana, D.V., Feng, H.C., Becker, J.C., jenkins, F.J., Moschos, S., Chang, Y., Buck, C.B., Moore, P.S., 2009. Human Merkel cell polyomavirus infection II. MCV is a common human infection that can be detected by conformational capsid epitope immunoassays. Int. J. Cancer 125, 1250–1256.

Türler, H., Beard, P., 1985. Simian virus 40 and polyoma virus: growth, titration, transformation and purification of viral components. In: mahy, B.W.J. (Ed.), Virology: a Practical Approach. IRL Press, Oxford.

Wessa, P., 2012. Spearman Rank Correlation (v1.0.1) in Free Statistics Software (v1.1.23-r7), Office for Research Development and Education, URL ⟨http://www.wessa.net/rwasp_spearman.wasp/⟩.

Yamaguchi, M., Depamphilis, M.L., 1986. DNA binding site for a factor(s) required to initiate simian virus 40 DNA replication. Proc. Natl. Acad. Sci. USA 83, 1646–1650.