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Clinical polyomavirus BK variants with agnogene deletion are non-functional but rescued by *trans*-complementation

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ABSTRACT

High-level replication of polyomavirus BK (BKV) in kidney transplant recipients is associated with the emergence of BKV variants with rearranged (rr) non-coding control region (NCCR) increasing viral early gene expression and cytopathology. Cloning and sequencing revealed the presence of a BKV quasispecies which included non-functional variants when assayed in a recombinant virus assay. Here we report that the rr-NCCR of BKV variants RH-3 and RH-12, both bearing a NCCR deletion including the 5′ end of the agnoprotein coding sequence, mediated early and late viral reporter gene expression in kidney cells. However, in a recombinant virus they failed to produce infectious progeny despite large T-antigen and VP1 expression and the formation of nuclear virus-like particles. Infectious progeny was generated when the agnogene was reconstructed *in cis* or agnoprotein provided *in trans* from a co-existing BKV rr-NCCR variant. We conclude that complementation can rescue non-functional BKV variants *in vitro* and possibly *in vivo*.

Introduction

Polyomavirus BK (BKV) infects more than 80% of the human population and establishes latency in the renourinary tract. In approximately 5–10% of healthy individuals BKV reactivates and gives asymptomatic low-level urinary shedding (Egli et al., 2009; Polo et al., 2004). However, reactivation in kidney or in bone marrow transplant recipients is associated with much higher urine BKV loads and progression to polyomavirus-associated nephropathy and hemorrhagic cystitis, respectively (Dropulic and Jones 2008; Hirsch and Steiger 2003).

BKV has a ~5000 basepairs circular double-stranded DNA genome that can be divided into a regulatory, an early and a late coding region. The regulatory or non-coding control region (NCCR) contains promoter/enhancer elements for the viral early and late genes and has been arbitrarily divided into five consecutive sequence blocks designated O142, P68, Q39, R63 and S63, where the numbers indicate the length in basepairs (Markowitz and Dynan, 1988; Moens et al., 1995). The most commonly found BKV strains in urine of immunocompetent individuals have a linear anatomy of the O-P-Q-R-S blocks which is denoted archetype or ww-NCCR (Egli et al., 2009; Rubinstein et al., 1987). In kidney transplant recipients, BKV strains with rearranged NCCRs (rr-NCCR) have been found in urine, plasma and allograft biopsies (Azzi et al., 2006; Chen et al., 2001; Gosert et al., 2008; Olsen et al., 2006; Perets et al., 2009; Randhawa et al., 2003; Sharma et al., 2007). The early region encodes the important regulatory large tumour antigen (LT-ag) and the supportive small T-ag (st-ag), while the late region encodes the structural proteins VP1, VP2, VP3 as well as the agnoprotein.

Agnoprotein is a small basic protein abundantly expressed in the cytoplasm during the late phase of the BKV life cycle, showing some enrichment in the perinuclear region (Rinaldo et al., 1998). This abundant expression pattern has also been observed *in vivo* in kidney biopsies from patients with polyomavirus-associated nephropathy (Leuenberger et al., 2007). The precise function of BKV agnoprotein is presently unknown. Recently, the BKV agnoprotein was found to co-localize with lipid droplets in the cytoplasm of Vero and primary human tubular epithelial cells (Unterstab et al., revision). Another study reports the detection of minute fractions of BKV agnoprotein in the nucleus (Johannessen et al., 2008). Reports on agnoprotein function in JCV and SV40 infection implicate a multitude of essential functions in viral gene expression, virion assembly, maturation and release (Khalili et al., 2005). The JCV agnoprotein has been reported to facilitate nuclear egress of virions by destabilizing the nuclear membrane through disrupting the interaction of HP1α with the lamin B receptor, a transmembrane protein in the inner nuclear membrane (Okada et al., 2005).

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In a previous study of BKV NCCR variants in urine and kidney allograft biopsies from kidney transplant recipients (Olsen et al., 2006), we detected two variants with rr-NCCRs and deletions in the 5′ part of the agnogene. NCCR variant RH-3 was found in a biopsy, while RH-12 was found in the urine of another patient. Of note, both clinical samples contained a number of additional NCCR variants with complete agnogene. To the best of our knowledge, naturally occurring variants without agnoprotein have not been reported for BKV, JCV or SV40; thus, we conducted a functional analysis of RH-3 and RH-12 variants in kidney cells.

Results

Characterization of RH-3 and RH-12

The promoter strength of RH-3 and RH-12 NCCR was first compared with the archetype ww-NCCR and the rearranged BKV (Dunlop) NCCR using the dual reporter gene expression vector phRG described by Gosert et al. (2008). Vero cells were transfected with phRG-RH-3, phRG-RH-12, phRG-WW and phRG-Dunlop, and the expression pattern examined 2 d p.t. The RH-3, RH-12 and BKV

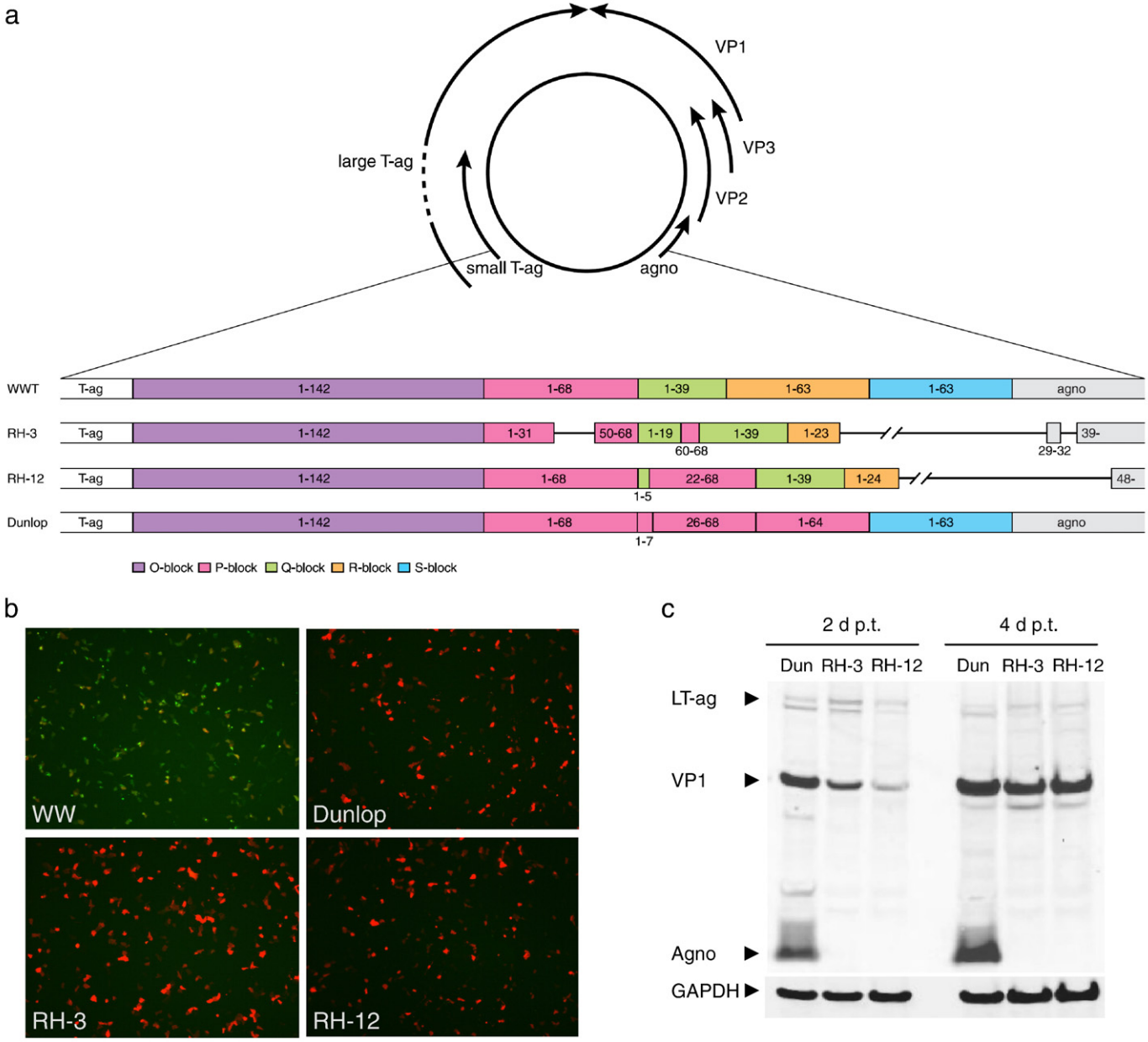

Fig. 1. Characterization of RH-3 and RH-12 amplified from kidney transplant recipients. (a) Schematic illustration of the genome of the recombinant virus RH-3 and RH-12. The PCR amplified *SacI* fragment containing the NCCR was cloned into the BKV(Dunlop) backbone replacing the Dunlop NCCR. The transcription factor binding sequence blocks are shown in different colours as indicated below. While archetype BKV NCCR WWT has one of each block and the complete agnogene, RH-3 and RH-12 have rearranged P- and Q-blocks and lack parts of the R-block, the S-block and the 5′ end of the agnogene. BKV(WWT) and BKV(Dunlop) were used as controls in most experiments. (b) NCCR activity. Vero cells 2 d p.t. with the dual-reporter plasmid phRG with green (GFP) and red fluorescence protein (RFP) expression under the control of WW, RH-3, RH-12 or BKV(Dunlop) NCCR. Red fluorescence simulates early gene expression while green fluorescence simulates late gene expression. The pictures are taken using a Nikon TE2000 microscope equipped and processed with NIS Elements Basic. Exposure time on all pictures: red 1/3 s and green 1 1/5 s. (c) BKV and cellular protein expression. Western blot on cell extracts from Vero cells 2 and 4 d p.t. with recombinant RH-3, RH-12 or BKV(Dunlop). Polyclonal rabbit anti-sera directed against LT-ag, VP1 or agnoprotein and a monoclonal mouse antibody directed against GAPDH were used as primary antibodies. The housekeeping protein GAPDH is shown as a loading control. (d) Subcellular localization BKV proteins. Immunofluorescence staining of Vero cells 4 d p.t. with recombinant RH-3, RH-12 or BKV(Dunlop). Monoclonal mouse antibody anti-SV40 LT-ag (red) and polyclonal rabbit anti-sera directed against VP1 (green) were used as primary antibodies. Cell nuclei were stained with DRAQ5 (blue). (e) Demonstration of virus-like particles. IEM of Vero cells 4 d p.t. with RH-3 or RH-12. Virus-like particles were labelled with polyclonal rabbit anti-sera directed against VP1 and protein-A-gold 10 nm (black dots).

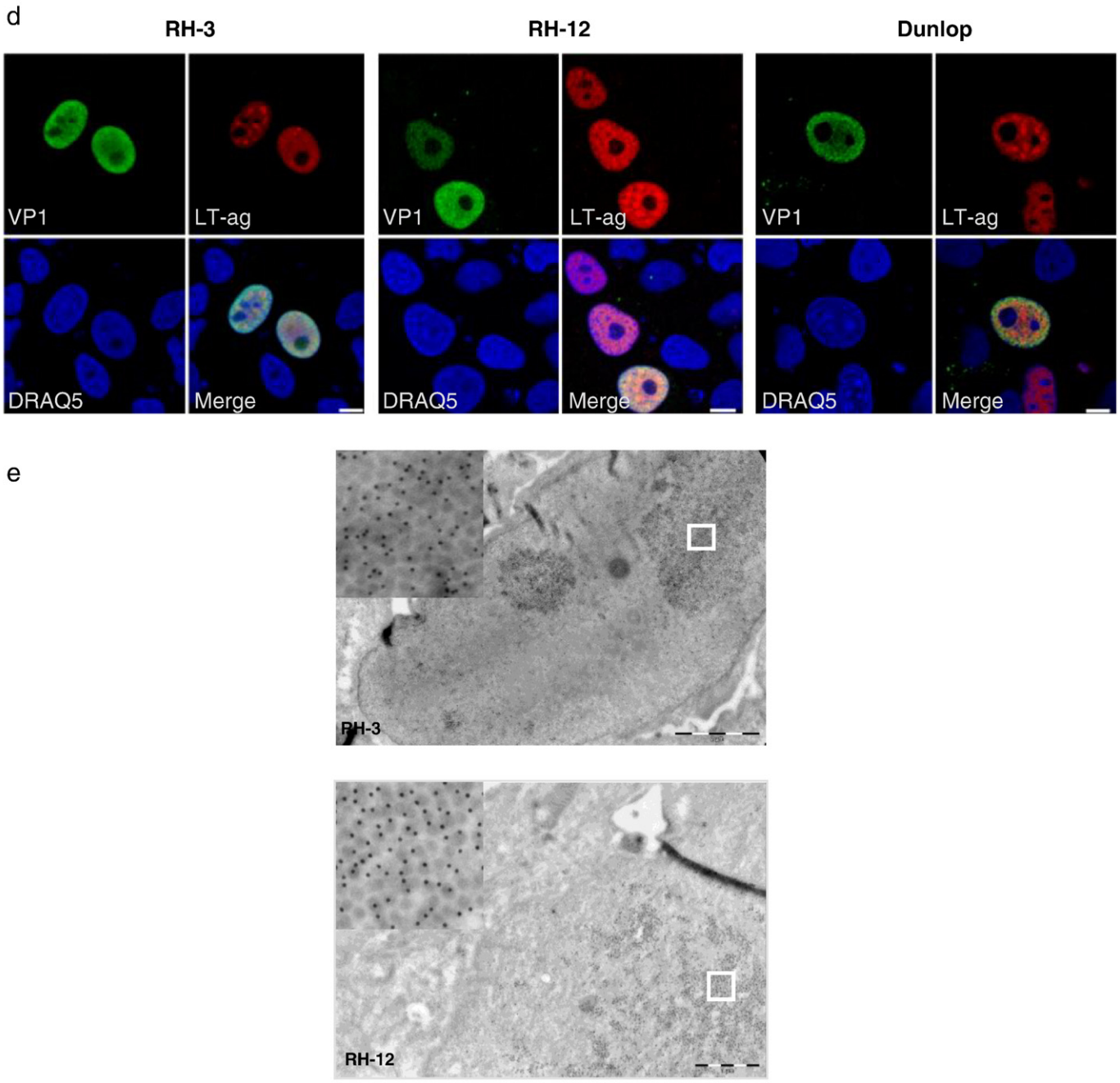

Fig. 1 (*continued*).

(Dunlop) NCCRs all demonstrated strong RFP expression and weak GFP expression while ww-NCCR showed the opposite pattern with weak RFP and strong GFP (Fig. 1b). These results indicate that RH-3, RH-12 and BKV(Dunlop) behave like other rr-NCCRs by having a strong early but weak late expression while ww-NCCR have the opposite expression pattern, as earlier demonstrated by Gosert et al. (2008). We concluded that RH-3 and RH-12 NCCRs were functional promoters with similar characteristics as previously described for other rr-NCCRs.

Next we investigated the expression of BKV proteins from RH-3 and RH-12 recombinant viruses. Vero cells were transfected with religated genomes, cell extracts harvested 2 and 4 d p.t. and cells were fixed 4 d p.t. Again BKV(Dunlop) was included as a positive control. Western blot demonstrated production of LT-ag and VP1 from all three strains, but only BKV(Dunlop) produced agnoprotein (Fig. 1c). This was confirmed on the single-cell level by immunofluorescence staining, which also demonstrated that RH-3 and RH-12 transfected cells have the same nuclear localisation of VP1 and LT-ag as BKV (Dunlop) (Fig. 1d).

To examine the ability of RH-3 and RH-12 recombinant viruses to produce infectious viral particles, Vero cell supernatants 4 d p.t. were seeded on permissive HUV-EC-C. When supernatants from RH-3 or RH-12 transfected cells were used as inoculum, immunofluorescence staining with VP1 and LT-ag antibodies 4 days post-infection (d p.i.) was negative while BKV(Dunlop) supernatant resulted in a high percentage of infected cells (data not shown). In order to find out if the nucleus of RH-3 and RH-12 transfected Vero cells contained only VP1 or also viral particles, immune electron microscopy with gold labelled VP1 antibodies was performed. Virus-like particles were detected in the nucleus of both RH-3 and RH-12 transfected cells (Fig. 1e).

To investigate whether nuclear virus-like particles were infectious but simply not released into the supernatant, RH-3, RH-12 and BKV (Dunlop) transfected Vero cells were subjected to three cycles of freezing and thawing to destroy cellular membranes and liberate viral particles. This lysate was then used to inoculate HUV-EC-C. Immunofluorescence staining with antibodies for VP1 and LT-ag 4 d p.i. only revealed stained cells in wells inoculated with BKV(Dunlop) lysate but not with RH-3 or RH-12 recombinants (data not shown).

We concluded that deletion in the 5′ part of the agnogene in RH-3 and RH-12 allowed for LT-ag and VP1 protein expression, but not for agnoprotein expression. LT-ag and VP1 showed the expected nuclear localization and VP1 assembled into virus-like particles. However, RH-3 and RH-12 did not release infectious progeny into cell culture supernatants, and the intranuclear virus-like particles turned out to be non-infectious when artificially released from the host cells.

Influence of agnoprotein given in trans to RH-3 and RH-12

To investigate whether providing agnoprotein *in trans* would allow for RH-3 and RH-12 to generate infectious viral particles, we co-transfected RH-3 or RH-12 together with three different genomic BKV plasmids corresponding to the archetype pWWT and the rr-NCCR BKV pRH-9 and pRH-7. In these three plasmids the VP1 gene was disrupted by the vector sequence, but the remaining viral genes including the agnoprotein coding sequence was left intact (Fig. 2a). Supernatants and cell extracts were harvested 4 d p.t. The cell extracts were analyzed by Western blot using antibodies against LT-ag, VP1 and agnoprotein. While single transfected RH-12 cells only produced LT-ag and VP1, co-transfected cells also expressed agnoprotein (Fig. 2b). Similar results were also obtained for co-transfections with RH-3 (data not shown). By contrast, no VP1 was detected in the control cells transfected with pWWT, pRH-9 or pRH-7 bearing the disrupted VP1 gene.

Then, the production of infectious particles in Vero cells was examined by seeding the supernatants onto HUV-EC-C. Immunofluorescence staining with antibodies directed against LT-ag and VP1, or LT-ag and agnoprotein, was performed 4 d p.i. This time, infection could be demonstrated in HUV-EC-C. Importantly, no agnoprotein but only LT-ag and VP1 was detected indicating that the infectious particles must have originated from RH-3 and RH-12 and not from the helper plasmid providing agnoprotein *in trans*. Supernatants obtained from co-transfection with pRH-7 gave rise to a higher number of BKV expressing cells, and therefore contained higher viral load than supernatants from co-transfection with pRH-9 or pWWT (Fig. 2c, and data not shown). Of note, the recombinant RH-7 was recently found to replicate better than the recombinant RH-9 and BKV WWT in both Vero cells and RPTECs (Olsen et al., 2009). We concluded that providing agnoprotein and LT-ag *in trans* complemented the deficiency of RH-3 and RH-12 to produce infectious viral particles. Since LT-ag and VP1 are made by RH-3 and RH-12, complementation of the absent agnoprotein was most likely to provide the necessary contribution.

The effect of replacing the truncated agnogene with a complete agnogene — characterization of RH-3-agno and RH-12-agno

To investigate directly whether the addition of agnoprotein would be sufficient for infectious virus production of RH-3 and RH-12, we reconstructed RH-3 and RH-12 with a complete agnoprotein coding sequence, denoted RH-3-agno and RH-12-agno. Vero cells were then transfected with RH-3-agno and RH-12-agno genomes using RH-3, RH-12 and BKV(Dunlop) as controls. Cell extracts were harvested 4 d p.t. and Western blot was performed using antibodies against LT-ag, VP1 and agnoprotein. As shown in Fig. 3a, both RH-3-agno and RH-12-agno expressed LT-ag, VP1 and agnoprotein. Immunofluorescence staining 4 d p.t. also demonstrated normal cytoplasmic agnoprotein distribution (Fig. 3b). The production of infectious progeny was examined by seeding supernatants from Vero cells 4 d p.t. on HUV-EC-C. Immunofluorescence staining with antibodies to LT-ag and agnoprotein revealed infected cells when supernatants from transfections with RH-3-agno (Fig. 3c), RH-12-agno (data not shown) or BKV (Dunlop) (data not shown) but not from RH-3 or RH-12 were inoculated onto HUV-EC-C. In agreement with this, qPCR on DNase I treated Vero cell supernatants 4 d p.t. only detected BKV load levels above input for RH-3-agno, RH-12-agno and BKV(Dunlop) (Fig. 3d).

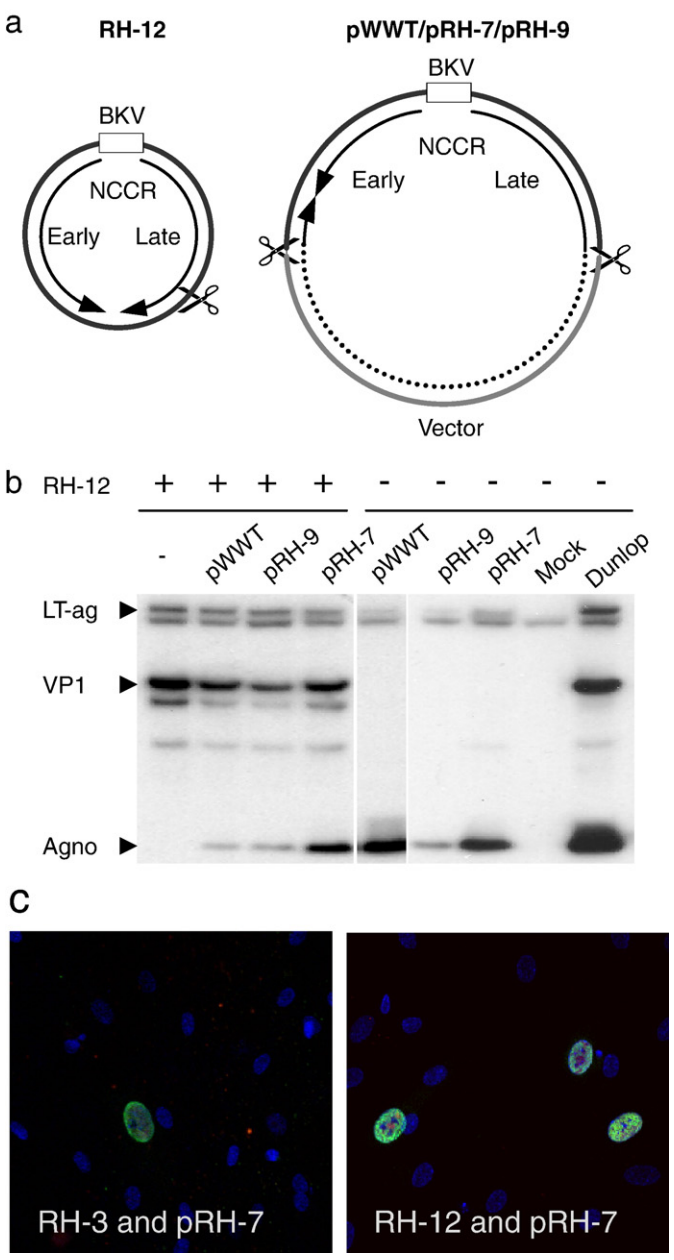

Fig. 2. *Trans*-complementation. (a) Schematic presentation of constructs. RH-12 was released from the vector and religated before transfection into Vero cells. The same was done for RH-3 (not shown). pRH-7, pRH-9 and pWW all contain the complete BKV genome in a vector. Since the genome is cloned via a restriction site in VP1 the VP1 gene is interrupted. (b) BKV protein expression. Western blot on cell extracts from Vero cells 4 d p.t. with RH-12 and the BKV genomes in vector (pWW, pRH-9 or pRH-7) or only the BKV genomes in vector. Polyclonal rabbit anti-sera directed against LT-ag, VP1 and agnoprotein were used as primary antibodies. (c) Detection of infectious progeny. Supernatants harvested 4 d p.t. from Vero cells co-transfected with RH-12 and pRH-7 or RH-3 and pRH-7 were seeded onto HUV-EC-C. Immunofluorescence staining with monoclonal mouse antibody anti-SV40 LT-ag (red) and polyclonal rabbit anti-serum directed against VP1 (green) was performed 4 d p.i. Cell nuclei were stained with DRAQ5 (blue).

We concluded that the presence of agnoprotein enabled RH-3 and RH-12 to produce and release infectious virus into the cell culture supernatant.

BKV agnoprotein and HP1α

To investigate whether or not BKV agnoprotein facilitated nuclear egress by interacting with proteins of the nuclear membrane as reported previously for JCV agnoprotein (Okada et al., 2005), we infected primary RPTECs with BKV(Dunlop) and stained for agnoprotein and lamin A/C. As shown in Fig. 4a and a′, we could not detect co-localization of both proteins. Lamin A/C staining was clearly nuclear while the agnoprotein staining was exclusively cytoplasmic. Interestingly, the staining pattern of lamin was different in BKV infected and uninfected cells. In infected cells, the majority of lamin accumulated at the nuclear rim while in uninfected cells, the lamin was present throughout the nucleoplasm (Fig. 4a, see arrowheads). Next, we investigated if agnoprotein and HP1α co-localized. HP1α was present throughout the nucleoplasm with the exception of some distinct unstained areas, but we detected no co-localization with agnoprotein (Fig. 4b and b′). Immunostaining with VP1 and HP1α revealed that the areas unstained by HP1α had strong VP1 staining, probably indicating areas of virion assembly (Fig. 4c). However, LT-ag and HP1α had a common staining pattern and partly overlapped in the VP1-free intranuclear areas (Fig. 4d). Immunostaining for lamin A/C and HP1α confirmed the co-localization of the two proteins at the outer nuclear rim (Fig. 4e). We concluded that the role of BKV agnoprotein in facilitating the generation and release of infectious progeny could not be explained by an interaction with the lamin A/C or HP1α in the nuclear membrane.

Discussion

The expression of BKV agnoprotein was demonstrated more than 10 years ago (Rinaldo et al., 1998), but still the exact function is unknown for this small abundant protein found in the cytoplasm of BKV infected cells *in vitro* and *in vivo* (Leuenberger et al., 2007).

The two BKV strains with rr-NCCR and deletion in the 5′ end of the agnogene were found in a urine sample (RH-12) and in a kidney allograft biopsy (RH-3) from different kidney transplant recipients. Reporter assay analysis of the rr-NCCRs showed promoter activities similar to the highly replicating BKV(Dunlop) strain, with a strong early but weak late promoter activity. Replacing the rr-NCCR of the BKV(Dunlop) with the corresponding rr-NCCR of RH-3 and RH-12 confirmed the functionality through production of both early LT-ag and late VP1 proteins. While the production of early proteins was similar to BKV(Dunlop), VP1 expression was slightly lower and there was no agnoprotein detectable. The absence of agnoprotein was coupled to the lack of infectious progeny, both intracellularly and extracellularly, suggesting that agnoprotein plays a role in the generation of infectious virus particles. Electron microscopy

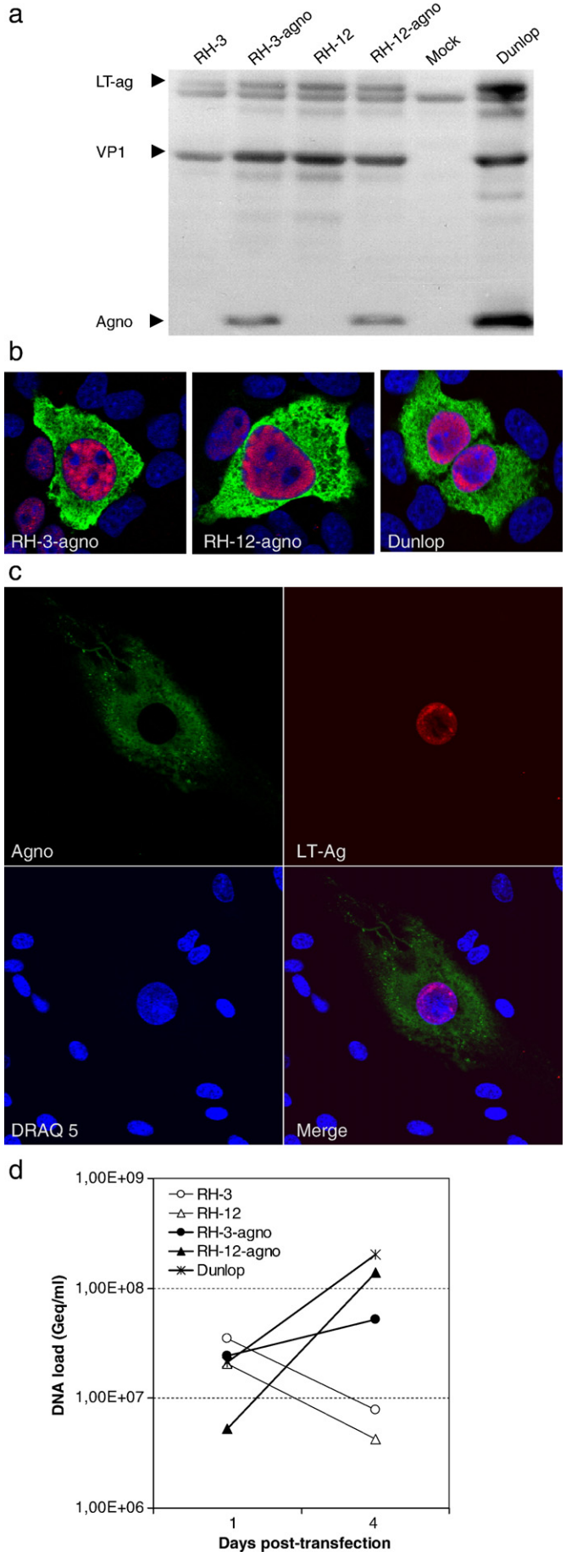

Fig. 3. Characterization of RH-3-agno and RH-12-agno. (a) BKV protein expression. Western blot on cell extracts from Vero cells 4 d p.t. with RH-3, RH-3-agno, RH-12, RH-12-agno, BKV(Dunlop) or mock. Polyclonal rabbit sera directed against LT-ag, VP1 and agnoprotein were used as primary antibodies. (b) Subcellular localization of BKV proteins. Immunofluorescence staining of Vero cells 4 d p.t. with RH-3-agno, RH-12-agno and BKV(Dunlop). Monoclonal mouse antibody anti-SV40 LT-ag (red) and polyclonal rabbit anti-serum directed against agnoprotein (green) were used as primary antibodies. Cell nuclei were stained with DRAQ5 (blue). (c) Demonstration of infectious particles. Supernatants from Vero cells 4 d p.t. with RH-3-agno were harvested and seeded onto HUV-EC-C. Immunofluorescence staining 4 d p.i. with monoclonal mouse antibody anti-SV40 LT-ag (red) and polyclonal rabbit anti-serum directed against agnoprotein (green) was used as primary antibodies. Cell nuclei were stained with DRAQ5 (blue). (d) Release of viral progeny. Quantitative PCR on DNase I treated supernatants from RH-3, RH-3-agno, RH-12, RH-12-agno and BKV(Dunlop) transfected Vero cells 1 and 4 d p.t. Data are presented as BKV load (Geq/ml). The DNA load at 1 d p.t. is input DNA.

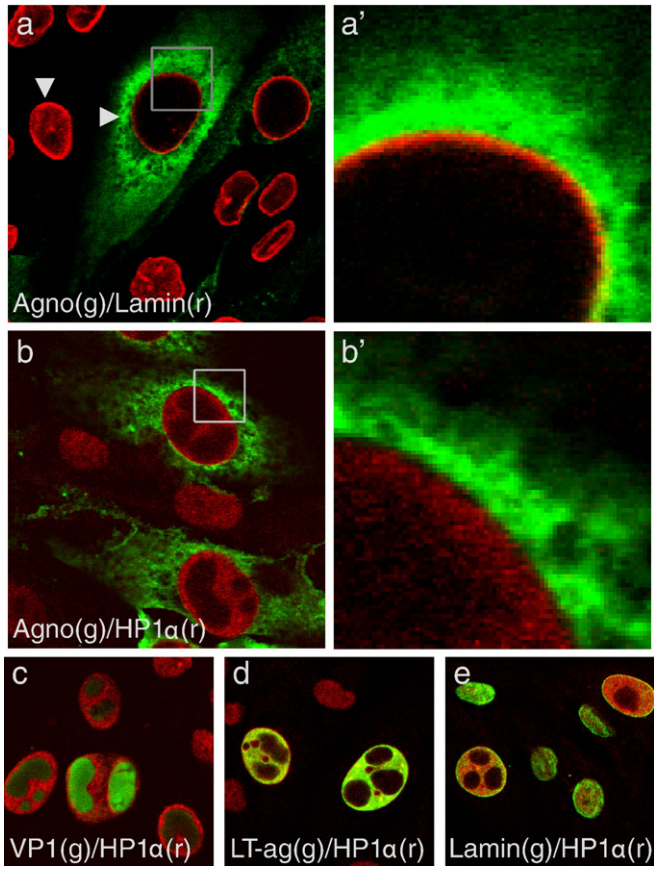

Fig. 4. Co-localization study of BKV agnoprotein with lamin A/C and HP1α. Immunofluorescence staining of RPTECs 3 d p.i. with BKV(Dunlop). The following primary antibodies were used: (a and a′) anti-agnoprotein and anti-lamin A/C (monoclonal), (b and b′) anti-agnoprotein and anti-HP1α, (c) anti-VP1 and anti-HP1α, (d) anti-LT-ag and anti-HP1α, (e) anti-lamin A/C (polyclonal) and anti-HP1α. Secondary antibody anti-rabbit conjugated with Alexa fluor 488 (giving green fluorescence) is labelled with (g), while anti-mouse conjugated Alexa fluor 568 (giving red fluorescense) is labelled with (r). In (a) the upper arrowhead indicate an uninfected cell while the lower arrowhead indicate an infected cell.

documented the presence of virus-like particles in Vero cells transfected with RH-3 or RH-12 DNA. Despite this similar morphology, infectivity was not found suggesting that other critical steps had not occurred. One such step could be packaging of the viral genome. In accordance with this, no viral DNA above input level could be detected in the cell culture supernatants by qPCR. For JCV, mutation of the agnoprotein phosphorylation sites was enough to prevent encapsidation of viral DNA (Sariyer et al., 2006). In contrast, this was not found when a phosphorylation site in BKV agnoprotein was mutated (Johannessen et al., 2008). For SV40 packing of viral DNA has been associated with VP2 and VP3 function (Roitman-Shemer et al., 2007). As SV40, BKV has two classes of late RNAs, 16S and 19S, which are generated by alternative splicing from a common pre-mRNA. While the 19S RNA is translated to yield both VP2 and VP3, the 16S RNA species is translated to yield VP1 and agnoprotein. The partially deleted agnogene should therefore not affect the expression of VP2 and VP3. However, studies of SV40 demonstrate that a minor fraction of agnoprotein is made from the 19S RNA, and that an open reading frame for agnogene reduce the synthesis of VP2 and VP3 three- to five-fold (Good et al., 1988). If this is the same for BKV, we would expect a slightly increased VP2 and VP3 expression from RH-3 and RH-12.

Addition of the complete agnoprotein coding sequence to RH-3 and RH-12 enabled RH-3-agno and RH-12-agno to produce infectious virus. Moreover, co-transfection with genomic BKV plasmids corresponding to the archetype pWWT or the rr-NCCR BKV pRH-9 and pRH-7, in which the VP1 gene was disrupted, provided agnoprotein *in trans* and allowed for completion of the BKV life cycle. This observation indicates that agnoprotein-deficient BKV genomes can be effectively rescued by co-transfection *in vitro*. Although our results suggest that BKV agnoprotein facilitate the assembly and/or maturation of infectious virions, release may also be affected. However the precise involvement in release must be different than for JCV agnoprotein, which is reported to act on the nuclear membrane. More importantly, our results suggest that *trans*-complementation by superinfection could be a mechanism to rescue non-functional BKV variants *in vivo*.

These are the only reported natural occurring BKV variants with deletion of the 5′ end of the agnoprotein coding sequence eliminating agnoprotein production. BKV(AS), a variant detected in the urine of a pregnant woman but first sequenced after several passages in cell culture, contain a deletion affecting the start codon of the agnogene (Tavis et al., 1989). However, Tavis et al. predicted an alternative in frame start codon located upstream in the S block. This start codon would give an agnoprotein with eight additional amino acids in the N-terminal part. By transfecting BKV(AS) DNA into Vero cells, we confirmed that BKV(AS) is able to express agnoprotein with normal cytoplasmic localization (Myhre and Rinaldo, unpublished data). Several BKV genomes with a deletion of nine nucleotides in the 3′ end of the agnogene have been reported in the urine of non-immunocompromised individuals (Yogo et al., 2008). No frame shifts were created; therefore these genomes could probably encode truncated agnoproteins with structural changes. Also a JCV variant with deletion in the 3′end of agnogene has been discovered in a urine sample (Jobes et al., 1999).

Agnoprotein deletion mutants have been reported for SV40 after serial passage in cell cultures at high multiplicities of infection. The SV40 mutants without agnoprotein expression replicate their DNA with wild-type kinetics but release mature virions inefficiently and as a consequence produce small plaques (Hou-Jong et al., 1987; Ng et al., 1985; Resnick and Shenk 1986). During the last years three additional human polyomavirus have been isolated: KI virus (KIV) and WU virus (WUV) from respiratory tract samples (Allander et al., 2007; Gaynor et al., 2007) and MC virus (MCV) from Merkel cell carcinoma (Feng et al., 2008). These new polyomaviruses have a genome organization similar to BKV and JCV but seem to lack an open reading frame corresponding to the agnogene. This indicates that they can sustain the life cycle without the agnoprotein. Recently, BKV(Dunlop) with an introduced point mutation in the agnogene start codon, preventing agnoprotein production, was found to produce infectious progeny in Vero cells but only at very low yields (Johannessen et al., 2008). This mutant had a 2-fold increased expression of both LT-ag and VP1 2 d p.t. of Vero cells. For RH-3 and RH-12, we did not find a higher LT-ag and VP1 expression compared to RH-3-agno and RH-12-agno at 4 d p.t. However, natural variants RH-3 and RH-12 differ from this constructed Dunlop mutant by lacking the first part of the agnogene and by containing a NCCR with different organization. Interestingly a point mutation in the JCV agnogene start codon was reported to negatively affect the expression of LT-ag and VP1 (Akan et al., 2006). Clearly, more detailed studies are needed to unravel the functional role(s) of agnoprotein.

When BKV NCCR activity was studied by the use of the bi-directional red–green reporter assay, both RH-3 and RH-12 NCCR were found to have promoter activities akin to the BKV(Dunlop) NCCR. Indeed, BKV(Dunlop) has a rr-NCCR and behaves similar to majority species rr-NCCR emerging in recipients with polyomavirus-associated nephropathy (Gosert et al., 2008) by showing a strong early, but weak late promoter activity. The corresponding recombinant BKV variants also showed increased LT-ag expression and the highest replication rates *in vitro*. Our results on the RH-3 and RH-12 clearly demonstrate that a deletion in agnoprotein coding sequence overrules the apparent advantageous NCCR and that a strong early promoter is not enough to

be successful, unless rescued by agnoprotein complementation *in trans*. *Trans*-complementation is not unique to BKV infection and has, for example, also been described for cytolomegalovirus where viral fitness was affected (Cicin-Sain et al., 2005).

In conclusion, we have found that natural BKV variants with deletions in the coding sequence of agnoprotein make virus-like particles but don't release infectious progeny into Vero cell supernatants. Addition of agnoprotein in *trans* or replacement of the truncated agnogene with a complete agnogene leads to release of infectious progeny. Our results suggest that BKV agnoprotein facilitates assembly and/or maturation of virions and that a possible involvement in release is different than suggested for JCV agnoprotein. They also imply that *trans*-complementation can rescue non-functional BKV variants *in vivo* which would explain their detection in a host with other BKV variants composing the BKV quasispecies.

Materials and methods

Cell cultures

Vero cells, derived from the kidney of an African green monkey (ATTC CRL 1587, www.atcc.org) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, www.invitrogen.com) containing 10% fetal bovine serum (Invitrogen). Human umbilical vein endothelial cells (HUV-EC-C, ATTC CRL 1730) were grown in Kaighn's FK12 medium (Invitrogen) containing 10% fetal bovine serum and endothelial cell growth factor (ECGF E-9640; Sigma, www.sigmaaldrich.com) according to the manufactures descriptions. Primary human renal tubule epithelial cells (RPTECs) (Lonza, www.lonzabioscience.com) were propagated as described by the manufacturer. All experiments were performed with RPTECs passage 4.

Viruses and plasmids

The generation of the recombinant viruses RH-3 (GenBank FJ940853) and RH-12 (GenBank FJ940861) from kidney transplant recipients is earlier described (Olsen et al., 2006). Briefly, RH-3 and RH-12 NCCRs (Fig. 1a) were amplified with primers 1 and 2 (Table 1) from a kidney allograft biopsy and urine sample, respectively. After SacI (NEB, www.NEB.com) digestion, the NCCR fragment was cloned into partial SacI digested pBKV(34-2) (BKV(Dunlop), ATTC 45025), thereby replacing the BKV(Dunlop) NCCR. The generation of RH-3 and RH-12 with complete gene encoding agnoprotein, RH-3-agno and RH-12-agno, was performed with gene splicing by overlap extension (SOE) (Horton et al., 1989). All the primers used are listed in Table 1. In short, RH-3 and RH-12 NCCR were PCR amplified by primers 1 and 3 (RH-3) or primers 1 and 5 (RH-12). Next, the first 145 nucleotides of the agnogene were amplified from pBKV(34-2) by primers 4 and 2 (RH-3) or primers 6 and 2 (RH-12) and subsequently joined to the RH-3 or RH-12 NCCR fragment, respectively, with a PCR using primers 1 and 2. The SacI NCCR fragment of RH-3-agno and RH-12-agno was finally cloned into pBKV(34-2) as described for RH-3 and RH-12. The integrity of the new plasmids was confirmed by sequencing. BKV(Dunlop) is known to replicate in Vero, RPTEC and HUV-EC-C (Bernhoff et al., 2008; Gosert et al., 2008; Grinde et al., 2007; Olsen et al., 2009), and was therefore used as a positive control. In order to make infectious viruses for transfection, the recombinant virus DNA was released from the vector by BamHI (NEB) digestion and religated with T4 DNA ligase (NEB). Two additional recombinant viruses, RH-7 and RH-9 (Olsen et al., 2006; Olsen et al., 2009), and the archetype WWT (GenBank M34048) were included in some experiments and the generation of these is as described for RH-3 and RH-12. These variants were only used incorporated into their vectors (pBR322 for RH-7 and RH-9, and pGEM3Zf(-1) for WWT) and then denoted pRH-7, pRH-9 and pWWT.

The generation of the dual-reporter plasmid phRG is earlier described (Gosert et al., 2008). In this plasmid the red fluorescent protein (RFP) and the green fluorescent protein (GFP) are in the same position as the early and late genes, respectively, and both are under control of the inserted BKV NCCR. To make phRG-RH-3 and phRG-RH-12, the NCCR sequences were amplified from the respective plasmids by PCR using primers 7 and 8 or 7 and 9. The MluI–BssHII-cleaved amplicon was then ligated into phRG digested with the same enzymes. As a positive control phRG-Dunlop was made by PCR using primer 7 and 10. The construct phRG-WW was made previously (Gosert et al., 2008).

Sequencing

Cycle sequencing was performed in both directions with primers 1 and 2 (Table 1) using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, www.appliedbiosystems.com). Sequencing reactions were analyzed on an ABI377 Prism Sequencer (Applied Biosystems).

Transient transfection

Transfection of Vero cells with religated RH-3 and RH-12 genomes or complete plasmids were done using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. Cells were transfected with 200 or 400 ng of religated BKV DNA per 0.8 cm^2 well (chamber slides) or 2 cm^2 well, respectively. For co-transfections of 2 cm^2 wells, 1200 ng of BKV plasmids (pWW, pRH-7 or pRH-9) and 400 ng of religated RH-3 or RH-12 DNA were used, giving a ratio of 3:1. Supernatants were collected 1, 2, and 4 days post-transfection (d p.t.), while cells were harvested 2 and 4 d p.t.

Quantitative PCR for BKV DNA

To quantitate extracellular BKV DNA, cell culture supernatants were harvested 1 and 4 d p.t. To remove uncapsidated DNA, DNase I (NEB) treatment was performed for 1 h followed by 5 min inactivation at 100°C. The supernatant was diluted 1:10 in dH_2O and quantitative BKV PCR was performed with primers and probes targeting the LT-ag (Hirsch et al., 2001).

Table 1
Primers.

No	Name	DNA sequence (5′–3′)	Size	Reference
1	GPPY1	CCAAAATCAGGCTGATGAGC	20	(Olsen et al., 2009)
2	GPPY2	TTCCCGTCTACACTGTCTTC	20	(Sundsfjord et al., 1994)
3	RH-3r	GCTGGCGCAGAACCATTGTGTTTATTTGAGAATTCTAGGGC	41	
4	RH-3f	AGAATTCTCAAATAAACACAATGGTTCTGCGCCAGCTGTC	40	
5	RH-12r	GCTGGCGCAGAACCATTTGTGTTTATTTGAGAATTCTAGGGC	42	
6	RH-12f	AGAATTCTCAAATAAACACAAATGGTTCTGCGCCAGCTGTC	41	
7	NCCR3	AAAAACGCGTCATTTTTGCAAAAATTGCAAAAGAATAGG	39	(Gosert et al., 2008)
8	RH-3NCCR4	TTTTGCGCGCCTGGCGCAGAACCATTGTGTTTA	33	
9	RH-12NCCR4	TTTTGCGCGCCTGGCGCAGAACCATTTGTGTTTA	34	
10	NCCR4	TTTTGCGCGCCTGGCGCAGAACCATGGCCTT	31	(Gosert et al., 2008)

Titration of viral infectivity

Titration of viral infectivity was performed on HUV-EC-C, which in contrast to Vero cells are highly permissive for BKV infection (Hanssen et al., 2005). Cell supernatants harvested 4 d p.t. from Vero cells were seeded onto HUV-EC-C. Two hours post-infection (p.i.), cells were washed once in PBS and complete medium was added. Four days p.i., cells were fixed and immunofluorescence staining conducted. In some experiments transfected Vero cells were subjected to three rounds of freezing and thawing before addition to HUV-EC-C.

Western blotting

Vero cells were harvested in 40 µl of lysis buffer (0.25 M dithiothreitol, 1:1 NuPAGE lithium dodecyl sulfate sample buffer, and double distilled H_2O), heated to 70°C for 10 min, and sonicated for 5 s. The samples were stored in −80°C until 17 µl protein lysate was separated by SDS PAGE (Invitrogen) and electrotransferred onto Millipore Immobilon-FL blotting-membrane. The detection of viral proteins was performed with monoclonal anti-SV40 LT-ag antibody (Ab-2 Pab416; 1:100, Chemicon, www.chemicon.com) or polyclonal rabbit antiserum directed against LT-ag (1:2000), VP1 (1:10,000), or agnoprotein (1:10,000) (Grinde et al., 2007; Hey et al., 1994; Rinaldo et al., 1998) and GAPDH (Ab8245; 1:5000, Abcam, www.abcam.com) as earlier described (Bernhoff et al., 2008).

Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde and permeabilised in methanol before blocking with 3% goat-serum/PBS followed by subsequently incubation with primary (37 °C) and secondary (r.t.) antibodies for 30 min. Primary antibodies were monoclonal anti-SV40 LT-ag antibody (Ab-2 Pab416) (1:100), HP1α (MAB3584; 1:1000, Chemicon) and Lamin A/C (Sc-7292; 1:100, Santa Cruz biotechnology, www.scbt.com) or rabbit polyclonal IgG Lamin A/C (Sc-20681; 1:100, Santa Cruz biotechnology) and polyclonal rabbit antiserum directed against agnoprotein or VP1(1:800). The secondary antibodies were anti-mouse conjugated with AlexaFluor 568 and anti-rabbit conjugated with AlexaFluor 488 (1:500; Molecular Probes, www.invitrogen.com). Nuclei were labelled with DRAQ5™ (Biostatus, www.biostatus.com). Images were taken using a Zeiss Axiovert 200 microscope (Carl Zeiss MicroImaging, www.zeiss.com) equipped with a LSM510 confocal module and processed using LSM5 software version 3.2 (Carl Zeiss MicroImaging, Inc.).

Immunoelectron microscopy (IEM)

Transfected Vero cells were fixed 4 d p.t. with 4% paraformaldehyde, washed in 0.12% glycin, scraped and pelleted in 12% gelantine. Next, the cell pellets were placed in 2.3 M sucrose overnight and subsequently cut in cubes, before mounted on cryo pins. The pellets were then frozen by immersion in liquid nitrogen and sectioned using a Leica EM UC6 Ultramicrotome. Sections were submerged in 1% cold water fish skin gelantine overnight. Finally, the sections were incubated with polyclonal rabbit serum directed against VP1 and then with protein-A-gold (10 nm). The specimens were contrasted with a mixture of uranyl acetate and methylcellulose and examined by a Jeol 1010 transmission electron microscope.

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