Blocks to herpes simplex virus type 1 replication in a cell line, tsBN2, encoding a temperature-sensitive RCC1 protein

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Circularization of the herpes simplex virus type 1 (HSV-1) genome is thought to be an important early event during the lytic cycle. Previous studies from another laboratory using a cell line, tsBN2, that carries a temperature-sensitive mutation in the gene encoding the regulator of chromatin condensation 1 (RCC1) indicated that functional RCC1 was required for HSV-1 genome circularization and subsequent viral DNA synthesis. Here, HSV-1 infection of tsBN2 cells has been re-examined by utilizing both wild-type HSV-1 and a derivative that enables a direct demonstration of circularization. At the non-permissive temperature, when RCC1 was absent, both circularization and viral DNA synthesis were reduced, but not abolished. However, no infectious progeny virus was detected under these conditions. An impairment in the cleavage of concatemeric DNA and the failure to express at least one capsid protein indicated that HSV-1 replication is also blocked at a late stage in the absence of RCC1. This conclusion was supported by a temperature-upshift experiment, which demonstrated a role for RCC1 at times later than 6 h post-infection. Finally, a virus constitutively expressing β-galactosidase produced the protein in a reduced number of cells when RCC1 was inactivated, suggesting that genome delivery to the nucleus or the initial stages of gene expression may also be affected.

INTRODUCTION

The majority of studies have found that the linear, double-stranded DNA genome of herpes simplex virus type 1 (HSV-1) circularizes upon entry into the cell nucleus prior to the establishment of latent or lytic infection (Efstathiou & Preston, 2005; Garber et al., 1993; Poffenberger & Roizman, 1985). Although a recent paper (Jackson & DeLuca, 2003) concluded that, during lytic infection, the initial templates for DNA synthesis were linear molecules, our subsequent analysis was in agreement with the earlier observations in demonstrating that circular genomes are generated rapidly upon infection, and function as the templates for replication (Strang & Stow, 2005).

To date, little is known about the viral DNA sequences and viral or cellular proteins required for circularization of the genome. A region of approximately 400 bp, the a sequence, is present as a direct repeat at the genomic termini and in inverted orientation at the junction between the L and S segments (Fig. 1a). Fusion of the a sequences at the opposite termini generates a novel sequence arrangement, Uc–DR1–Ub, as illustrated in Fig. 1(b). This element of approximately 200 bp, in which Uc and Ub originate from the L and S termini, respectively, represents the minimal packaging signal for replicated DNA and is sufficient to direct the cleavage of unit-length genomes from the concatemeric DNA-replication products and their insertion into pre-assembled procapsids to generate the DNA-containing capsid (Brown et al., 2002; Nasseri & Mocarski, 1988; Strang & Stow, 2005). The recovery of viable viruses following the replacement of all copies of the a sequence by a single Uc–DR1–Ub element suggests that any DNA sequences specifically required for genomic circularization are also likely to reside within this region (Strang & Stow, 2005).

Genome circularization has been proposed to occur by either direct ligation of the termini (Davison & Wilkie, 1981; Mocarski & Roizman, 1982; Strang & Stow, 2005) or by homologous recombination (Yao & Elias, 2001; Yao et al., 1997). To date, only one cellular protein species, regulator of chromatin condensation 1 (RCC1), has been implicated in the process (Umene & Nishimoto, 1996).

RCC1 acts as the guanine nucleotide-exchange factor for the Ran system, mediating the exchange of GDP for GTP on the small nuclear GTPase Ran (Bischoff & Ponstingl, 1991a, b; Drivas et al., 1990). The Ran system plays a pivotal role in nucleocytoplasmic transport, but also appears to be involved in other cellular activities, including microtubule function, nuclear-envelope assembly and entry into mitosis (Gorlich & Kutay, 1999; Moore, 2001; Nakielny & Dreyfuss, 1999). The absence of RCC1 disrupts the Ran system and...
therefore has the potential to affect many different cellular functions and regulatory pathways (Moore, 2001).

The importance of RCC1 was first uncovered in tsBN2 cells, a derivative of the baby hamster kidney cell line BHK-21 containing a temperature-sensitive mutation in the RCC1 coding sequence, which resulted in degradation of the RCC1 protein at the non-permissive temperature (NPT) of 39.5 °C (Nishimoto et al., 1978; Nishitani et al., 1991; Ohtsubo et al., 1987). Replication of HSV-1 in tsBN2 cells at the permissive temperature (PT; 33.5 °C) and NPT was investigated by Umene & Nishimoto (1996), who found that, in the absence of RCC1, viral DNA synthesis and progeny production could not be detected. Restriction-endonuclease fragments of the genome corresponding to the genomic termini and the L–S junction were analysed by Southern blotting in the presence and absence of the nuclease ATP–DNase, which digests linear and single-stranded circular DNA, but not double-stranded circular DNA. Higher levels of terminal fragments were detected early after virus infection at 39.5 °C than at 33.5 °C, and the viral DNA in cells infected at 39.5 °C was susceptible to ATP–DNase degradation. It was therefore concluded that an inability of the viral genome to circularize in the absence of RCC1 was a major factor in the lack of virus growth observed.

The ATP–DNase assay, however, represents an indirect assessment of HSV-1 genome circularization and circular molecules might remain susceptible to digestion due to the presence of nicks and gaps, which are known to occur in virion DNA (Frenkel & Roizman, 1972; Hyman et al., 1977; Wilkie, 1973). Importantly, although replicated HSV-1 DNA in the permissively infected parental cells was shown to be partially resistant to ATP–DNase, no similar analysis was performed prior to the initiation of viral DNA synthesis and it remains unknown whether circularized genomes resistant to the enzyme would be detectable under these conditions.

We previously described a derivative of HSV-1, HSV-1 P21, in which the viral a sequences had been replaced by the minimal Uc–DR1–Ub packaging element located in the viral thymidine kinase gene. In contrast to wild-type (wt) HSV-1, fusion of the termini of HSV-1 P21 produces a novel fragment in circular or concatemeric DNA, which is readily detected by Southern blot hybridization (Strang & Stow, 2005). This virus has now been used to re-examine genome circularization in tsBN2 cells. Our results indicated that this process is reduced, but not abolished, at NPT. The failure to detect infectious progeny virus under these conditions therefore suggested the possibility of further impairments to growth and subsequent investigations revealed that, although circular genomes could function as templates for DNA synthesis, a significant block occurred prior to assembly of the DNA-containing capsid.

**METHODS**

**Cells and viruses.** BHK-21 clone 13 (BHK) and Vero cells were grown as described previously (Strang & Stow, 2005). The tsBN2 line, containing a temperature-sensitive mutation in the RCC1 gene, was derived from BHK cells (Nishimoto et al., 1978; Uchida et al., 1990), and tsBN2-A1 cells are a ts+ transformant of tsBN2, stably transfected with human RCC1 cDNA (Umene & Nishimoto, 1996). tsBN2 and tsBN2-A1 cells were cultured at 32 and 37 °C, respectively, in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 100 U penicillin ml⁻¹, 100 μg streptomycin ml⁻¹, 1 x L-glutamine and 1 x minimal essential amino acids. All media were supplied by Invitrogen. In most experiments, 39.5 °C was employed as NPT for tsBN2 cells.

Stocks of wt HSV-1 strain 17 syn+ (McGeoch et al., 1988) and its derivative HSV-1 P21 (Strang & Stow, 2005) were prepared from the
HSV-1 with the lesion (C. Preston, personal communication) and is equivalent to wt HSV-1 growth in BHK, tsBN2 and tsBN2-A1 cells.

Virus-growth assay. Cell monolayers in 35 mm dishes were preincubated for 24 h at 32 °C (filled bars), 38.5 °C (empty bars) or 39.5 °C (shaded bars). The cells were maintained at these temperatures for a further 24 h and the yields of progeny virus were determined by titration on Vero cell monolayers. The arrow indicates that the yield from tsBN2 cells incubated at 39.5 °C was below the limit of detection (∼10^2 p.f.u. ml^-1).

DNA analysis. Total cell DNA was isolated as described previously (Strang & Stow, 2005). Cycloheximide was used at 200 μg ml^-1, as indicated, and was present from 1 h before and at all stages during infection.

Gel analysis of the DNAs was performed as described elsewhere (Stow, 2001; Stow & McMonagle, 1983). Samples of DNA corresponding to the yield from 2 × 10^6 cells were cleaved with BamHI and the resulting fragments were separated by agarose-gel electrophoresis. After transfer to a Hybond-XL membrane (Amersham Biosciences), viral DNA was detected by hybridization to a 32P-labelled probe prepared by nick translation of plasmid pGX2, containing the HSV-1 BamHI K fragment, or pGX153, containing the HSV-1 BamHI P fragment. Phosphorimages of Southern blots were acquired by using a Personal Molecular Imager and analysed with Quantity One software (both from Bio-Rad).

Western blot analysis. Western blotting was carried out as described previously (Strang & Stow, 2005). Primary antibodies, all used at a dilution of 1:1000 in TBSTM [10 mM Tris/HCl (pH 7.6), 150 mM NaCl, 0.1% Tween 20 and 5% dried milk], were anti-RCC1 antiserum KAM-CC225 (Stresa), anti-ICP0 antiserum 11060 (Everett et al., 1991), anti-UL38 antiserum 02040 (Adamson et al., 2006), anti-HSV-1 DNA polymerase antiserum 13429 (Marsden et al., 1994) and anti-β-actin antiserum (Sigma). The anti-RCC1 antiserum detects both the human and hamster RCC1 proteins.

RESULTS AND DISCUSSION

HSV-1 growth is impaired in the absence of RCC1

In an initial series of experiments, growth of wt HSV-1 was examined in tsBN2, tsBN2-A1 and BHK cells at 32, 38.5 and 39.5 °C, and a representative result is shown in Fig. 2. Similar yields were observed in tsBN2 and BHK cells at 32 °C. However, following incubation of tsBN2 cells at 39.5 °C, the temperature at which RCC1 has been reported to be degraded (Uchida et al., 1990), progeny virus was undetectable, corresponding to a >100 000 fold reduction in yield compared with BHK cells. Growth in tsBN2 cells at 38.5 °C was reduced by approximately 30-fold in comparison with BHK cells. Similar yields were obtained following incubation of tsBN2-A1 cells at all three temperatures, but these were consistently lower than those observed in BHK cells. Uninfected cells were assayed by Western blotting for the presence of RCC1 at the same temperatures (Fig. 3). In tsBN2 cells, RCC1 was present in reduced amounts at 38.5 °C compared with 32 °C and was undetectable at 39.5 °C. RCC1 was detected in BHK and tsBN2-A1 cells at all three temperatures, although there was some variation between the levels, with apparently increased expression in tsBN2-A1 cells at 32 °C compared with 38.5 or 39.5 °C. The levels of RCC1 in BHK cells were slightly elevated at the higher temperatures.

These results confirm the observation of Umene & Nishimoto (1996) that HSV-1 is unable to grow in tsBN2 cells.
cells at 39.5 °C, and indicate that the absence of RCC1 is a major factor contributing to this defect. In assessing the data, it should be noted that tsBN2 cells were isolated following chemical mutagenesis of the parental line, and it is quite possible that they may harbour other mutations that have small effects on virus growth. In addition, the ts+ transformant, tsBN2-A1, expresses the human form of RCC1. The fact that this protein is from a heterologous species, and is not necessarily expressed at an optimal level, may explain why HSV-1 replicates less efficiently in tsBN2-A1 cells than in wt BHK cells at all three temperatures. The reasons for the variation in RCC1 expression in tsBN2-A1 and BHK cells is not known, although the elevated levels observed in tsBN2-A1 cells at 32 °C may be, in part, due to the fact that both the endogenous hamster gene and inserted RCC1 cDNA are likely to be expressed efficiently at this temperature. Comparison of HSV-1 growth in tsBN2-A1 cells at the three temperatures indicates that the increased RCC1 levels are not detrimental to virus replication.

**HSV-1 DNA synthesis in the absence of RCC1**

It was suggested by Umene & Nishimoto (1996) that the major factor preventing virus growth in the absence of RCC1 is inhibition of viral DNA synthesis, caused by failure of the viral genome to circularize. To investigate this further, viral DNA synthesis was examined following incubation of BHK, tsBN2 or tsBN2-A1 cells infected with wt HSV-1 at 32 or 39.5 °C. Total DNA was prepared, cleaved with BamHI and hybridized to a labelled probe containing the BamHI K fragment, which detects the junction fragment, K, and terminal fragments, Q and S (Fig. 1a). The results are shown in Fig. 4.

Viral DNA replication was detected in all three cell lines at 32 °C, although tsBN2 and tsBN2-A1 cells exhibited some impairment, particularly at 4 h post-infection (p.i.) (Fig. 4a). In all three cell lines, fragments Q and S, as well as K, were observed at 24 h p.i., indicating that progeny genomes had been cleaved from concatemeric DNA. At 39.5 °C (Fig. 4b), efficient DNA synthesis and cleavage of concatemeric DNA occurred in both BHK and tsBN2-A1 cells, although the onset of DNA replication was delayed in the latter. In contrast, DNA synthesis in tsBN2 cells was reduced by approximately 30-fold and the terminal fragments, Q and S, were undetectable.

These results demonstrate that limited DNA synthesis can occur in the absence of RCC1 and that the reduced accumulation of viral DNA cannot alone account for the 100 000-fold reduction in virus yield from tsBN2 cells incubated at 39.5 °C. Cleavage of unit-length genomes from concatemeric DNA is coupled tightly to packaging of the DNA into capsids (Brown et al., 2002), and the failure of terminal fragments to accumulate in tsBN2 cells incubated at 39.5 °C suggests that there is a defect in the assembly of DNA-containing capsids. Interestingly, tsBN2-A1 cells were less impaired in DNA synthesis (Fig. 4) than in virus yield (Fig. 2) when compared with BHK cells. This may indicate that expression of the human RCC1 protein in these cells may have a detrimental effect on late events involved in the generation of infectious virions.

**Genome circularization in the absence of RCC1**

The virus HSV-1 P21 was employed in order to investigate directly the circularization of input genomes in tsBN2 cells. As illustrated in Fig. 1(c), this virus contains a single packaging signal within the U1 region and generates a novel junction fragment (J) when the two terminal fragments (T1 and T2) become fused. We demonstrated previously that fusion of the HSV-1 P21 termini occurs rapidly following infection in the presence of inhibitors of viral protein or DNA synthesis, and provided evidence that the observed end joining represents circularization rather
than concatemerization of the input genomes (Strang & Stow, 2005). tsBN2 cells were infected with HSV-1 P21 for 6 h at 32 and 39.5 °C in either the presence or absence of cycloheximide. DNA was prepared and cleaved with BamHI, and terminal and junction fragments were detected by hybridization to a probe containing the HSV-1 BamHI P fragment. A sample of the virus inoculum was analysed in parallel. The results are shown in Fig. 5.

As reported previously, a small amount of fragment J was detected in the virus inoculum (Fig. 5, lane 4), perhaps representing a low frequency of defective genomes or packaged circular molecules (Strang & Stow, 2005). Infection in the presence of cycloheximide blocks viral DNA synthesis, allowing the fate of input genomes to be investigated. Under these conditions, the amount of junction fragment detected was increased (Fig. 5, compare lanes 2 and 6 with lane 4). This increase occurred following incubation at both PT and NPT, but was consistently greater at 32 °C. The proportion of genomes with fused ends was calculated by expressing the counts in the novel junction fragment as a percentage of the counts in the two termini plus the junction and is shown for each lane in Fig. 5. These results demonstrate that circularization of HSV-1 P21 genomes is impaired, but not abolished, in the absence of RCC1.

Amplification of the junction fragment occurred in the absence of cycloheximide, suggesting that, at both temperatures, the resulting circular genomes can function as templates for DNA synthesis (Fig. 5, lanes 1 and 5), although, in agreement with the data presented in Fig. 4, this process is impaired at 39.5 °C. It should also be noted that the exposure time for the phosphorimage presented in Fig. 5 was chosen to show input genomes, whilst a shorter exposure was used to illustrate replicated viral DNA in Fig. 4.

### Genome delivery in the absence of RCC1

The reduction in circular genomes observed in the absence of RCC1 might result from inefficient delivery of the genome into the nucleus. To address this question, virus gene expression was assayed by titrating an HSV-1 strain, in1863, carrying a constitutively expressed lacZ marker gene, on each cell line at 32 and 39.5 °C (Table 1). The cells were stained for β-galactosidase expression and the titres were expressed as β-galactosidase-expressing particles (ml virus stock)−1 (Table 1).

Incubation of tsBN2 cells at 39.5 °C resulted in a decrease in titre of approximately twofold compared with incubation at 32 °C. In contrast, the titres recorded in BHK and tsBN2-A1 cells at 39.5 °C were approximately twofold and threefold greater, respectively, than those at 32 °C. This result confirms that HSV-1 genomes can be delivered to the nucleus in the absence of RCC1, but suggests that the process may be impaired to a small extent. It is possible that this contributes to the reduction in genome circularization observed under these conditions (Fig. 5). These results are consistent with observations that binding of purified HSV-1 capsids to the nuclear pores of isolated rat liver-cell nuclei requires a supply of hydrolysable Ran–GTP (Ojala et al., 2000), and suggest that RCC1 may have only an indirect effect on genome circularization.

### A late block to HSV-1 infection in tsBN2 cells

The detection of viral DNA synthesis, but not infectious progeny, in tsBN2 cells at 39.5 °C suggests that late stages of the viral lytic cycle may also be affected by the absence of RCC1. To examine this further, a temperature-upshift experiment was performed. Monolayers of tsBN2 cells were infected with 5 p.f.u. wt HSV-1 per cell following preincubation at 32 or 39.5 °C. After infection, two sets of

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**Fig. 5.** End joining in tsBN2 cells infected with HSV-1 P21. Monolayers of tsBN2 cells were mock-infected (Mock; lane 3) or infected with 5 p.f.u. HSV-1 P21 per cell (lanes 1, 2, 5 and 6) after 24 h preincubation at the indicated temperatures. Following adsorption for 1 h, the inoculum was removed and the cells were washed prior to continuation of incubation for a further 5 h at the same temperature. The cells in lanes 1 and 5 (No inhib.) were maintained in the absence of inhibitors, and those in lanes 2 and 6 were maintained in the presence of 200 μg cycloheximide (CHI) ml−1 present from 1 h before and throughout infection. Lane 4 (Input) corresponds to lysed uninfected cells spiked with the virus inoculum. DNA was prepared, and samples were cleaved with BamHI and analysed by agarose-gel electrophoresis and Southern blot hybridization with 32P-labelled pGX153 containing the BamHI P fragment. A phosphorimage of the washed membrane was acquired by using a Personal Molecular Imager (Bio-Rad). The positions of the two terminal fragments, T1 and T2, and the novel junction fragment, J, are indicated. The percentage of genome circularization was calculated as described in the text and is indicated in parentheses.
plates were incubated at the original temperature for 24 h. A third set of infected plates, preincubated at 32 °C, was maintained at this temperature for 6 h to allow genome circularization and the initiation of DNA synthesis to occur, and then shifted to 39.5 °C for a further 18 h. The yields of progeny virus were determined by titration on Vero cell monolayers, and mean ± SD values were calculated from four independent experiments. Titres of <10^2 and (9.0 ± 6.2) × 10^6 p.f.u. ml^{-1} were obtained from the cells maintained at 39.5 °C, respectively, whilst the corresponding value for the cells shifted to NPT at 6 h p.i. was (1.8 ± 0.7) × 10^5 p.f.u. ml^{-1}. This 50-fold reduction in yield following temperature upshift demonstrates that RCC1 must additionally play an important function late during virus infection.

Viral protein production in tsBN2 cells

Umene & Nishimoto (1996) reported that expression of two genes involved in viral DNA synthesis (ICP8 and DNA polymerase) was undetectable in the absence of RCC1. However, as we observed viral DNA replication under these conditions (Figs 4, 5), Western blotting was used to investigate the expression of representative immediate-early, early and late viral protein levels following infection of tsBN2 cells at 32 and 39.5 °C.

The immediate-early protein ICP0 and the early protein DNA polymerase were both detected following incubation of BHK, tsBN2 and tsBN2-A1 cells at 32 and 39.5 °C (Fig. 6). Both proteins were, however, less abundant in tsBN2 and tsBN2-A1 cells than in BHK cells following incubation at 39.5 °C. It was noted that a higher-molecular-mass protein was also detected specifically in infected cells by the anti-polymerase antiserum. This protein has been observed elsewhere (Yager et al., 1990), but it remains unclear whether it represents a modified form of DNA polymerase.

In contrast, the late protein VP19C, encoded by UL38, was expressed in all three cell lines following incubation at 32 °C, but could not be detected in tsBN2 cells infected at NPT. Given that this protein is a major structural component essential for capsid assembly and that cleavage of the genome is coupled tightly to insertion of the DNA into the capsid (Brown et al., 2002; Pertuiset et al., 1989), the absence of VP19C undoubtedly contributes to the observed failure to produce infectious progeny or to cleave concatemeric DNA at NPT (Figs 2, 4).

Conclusions

In agreement with the previous report of Umene & Nishimoto (1996), the presence of RCC1 was found to be

### Table 1. Expression of β-galactosidase in BHK, tsBN2 and tsBN2-A1 cells infected with in1863

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<tr>
<th>Cell line</th>
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<td>BHK</td>
<td>32.0</td>
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<tr>
<td>BHK</td>
<td>39.5</td>
<td>2.2 × 10^7</td>
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<td>tsBN2</td>
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<td>tsBN2</td>
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<tr>
<td>tsBN2-A1</td>
<td>32.0</td>
<td>1.7 × 10^6</td>
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<tr>
<td>tsBN2-A1</td>
<td>39.5</td>
<td>5.1 × 10^6</td>
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Fig. 6. Expression of viral proteins in BHK, tsBN2 and tsBN2-A1 cells. Cell monolayers were preincubated at either 32 °C (a) or 39.5 °C (b) followed by mock infection (lanes 1, 3 and 5) or infection with 5 p.f.u. wt HSV-1 per cell (lanes 2, 4 and 6). The cells were maintained a further 24 h at these temperatures prior to harvesting. Samples representing 5 × 10^4 cells were analysed by SDS-PAGE and Western blotting utilizing antisera specific for ICP0, Pol, UL38 and β-actin. The positions of protein size markers (kDa) are indicated on the right. HSV-1 DNA polymerase is indicated by an asterisk.

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Blocks to HSV-1 replication in tsBN2 cells
essential for HSV-1 growth. Our analysis, however, differs from theirs regarding the stages of the infection process that are affected. In particular, although both viral genome circularization and DNA synthesis were impaired, these activities were nevertheless detectable in tsBN2 cells infected at NPT. The reason for this difference is not known, and it is unclear whether the use of different HSV-1 strains or differences between the experimental approaches might be responsible. Although it is difficult to exclude the possibility that some residual RCC1 activity may remain in tsBN2 cells incubated at 39.5°C, allowing limited genome circularization and DNA synthesis to occur, our results indicate that depletion of RCC1 impacts upon additional events during the HSV-1 lytic cycle.

When infections were performed with an HSV-1 derivative that expresses β-galactosidase constitutively, enzyme activity was present in fewer cells when RCC1 was undetectable (Table 1). This suggests that an early stage in the infection process, such as delivery of genomes to the nucleus, may be affected, and this could result indirectly in the observed reduction in genome circularization. In addition, the results of a temperature-shift experiment demonstrated that RCC1 had an important, previously unrecognized function at times later than 6 h.p.i. Although other late proteins were not examined, the failure to express VP19C suggests that the transition to late-gene expression is possibly impaired. This might result from RCC1 playing a direct or indirect role in the late-gene transcription process, or possibly indicates that the DNA synthesized in the absence of RCC1 is an inefficient template. Regardless of the mechanism, the failure to express one or more late proteins essential for virion assembly is sufficient to explain the absence of cleavage of concatemeric DNA and production of infectious progeny.

In summary, the absence of RCC1 can clearly impact upon several processes occurring during the HSV-1 lytic cycle. Given the pivotal role that this protein plays in many cellular functions, this is perhaps not unduly surprising. For example, it can readily be envisaged that impairment of Ran-GTP-dependent nucleocytoplasmic transport in HSV-1-infected cells might affect genome entry into the nucleus, as well as nuclear import of viral proteins involved in transcriptional regulation, DNA synthesis, capsid assembly and DNA packaging. Moreover, effects on the transport of cellular cargoes are also likely to influence virus replication. A key challenge will be to distinguish direct from indirect consequences and to elucidate the mechanisms involved.

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