Virus particles produced by the herpes simplex virus type 1 alkaline nuclease null mutant ambUL12 contain abnormal genomes

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Open reading frame UL12 of herpes simplex virus type 1 (HSV-1) encodes an alkaline nuclease that has previously been implicated in processing the complex, branched, viral DNA replication intermediates and allowing egress of DNA-containing capsids from the nucleus. This report describes experiments using the HSV-1 UL12 null mutant ambUL12, which aim to explain the approximately 200- to 1000-fold decrease in the yield of infectious virus, compared with wild-type (wt) HSV-1, from non-complementing cells. A detailed examination revealed that both DNA replication and encapsidation were affected in ambUL12-infected cells, resulting in an approximately 15- to 20-fold reduction in the amount of packaged DNA. In contrast to previous reports, the absence of UL12 function did not greatly impair capsid release into the cytoplasm, and virus particles were readily detected in the supernatant medium from ambUL12-infected cells. The released virus, however, exhibited much higher particle/p.f.u. ratios than wt HSV-1, and this made a further important contribution to the overall reduction in yield. Gel analyses of packaged ambUL12 and wt DNAs revealed the presence of structural abnormalities. The DNA obtained from extracellular ambUL12 virions was non-infectious in transfection assays, and both ambUL12 DNA and virus particles exerted a dominant inhibitory effect on the growth of wt virus. These results suggest that ambUL12 virions produced in non-complementing cells have a greatly reduced ability to initiate new cycles of infection, and that this defect results from the encapsidation of abnormal genomes.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) contains a double-stranded DNA genome, 152 kbp in length. Origin-dependent viral DNA replication occurs in the nucleus of infected cells and requires the functions of seven essential proteins encoded by genes UL5, UL8, UL9, UL29, UL30, UL42 and UL52. The products of DNA synthesis are complex, branched, high molecular mass concatamers consisting of tandem head-to-tail repeats of the viral genome. DNA encapsidation proceeds by a mechanism in which site-specific cleavage of the concatamers into unit lengths is tightly coupled with their insertion into a preformed icosahedral procapsid. Cleavage and packaging require the products of the essential HSV-1 genes UL6, UL15, UL17, UL25, UL28, UL32 and UL33 (for reviews see Boehmer & Lehman, 1997; Boehmer & Nimonkar, 2003; Brown et al., 2002; Homa & Brown, 1997). Insertion of the genome into the procapsid is associated with the loss of an internal protein scaffold and conversion into a more angular form, the DNA-containing C capsid. Two other angularized capsid forms, the empty A capsid and the scaffold-containing B capsid, lack DNA and are believed to be dead-end products. C capsids, which represent the precursors of infectious virus particles released into the cytoplasm and extracellular medium, mature through the acquisition of a proteinaceous tegument layer and glycoprotein-containing lipid envelope (Brown et al., 2002; Homa & Brown, 1997).

In addition to the proteins described above, HSV-1 also encodes an alkaline nuclease, the product of the UL12 gene, which acts upon the viral DNA during the replication and packaging processes (Martinez et al., 1996a). Although the alkaline nuclease is not absolutely essential for virus replication in tissue culture, HSV-1 null mutants produce 100- to 1000-fold less infectious virus than their wild-type (wt) parents, and require complementing cell lines for efficient propagation (Gao et al., 1998; Martinez et al., 1996b; Patel et al., 1996; Weller et al., 1990). The nuclease activity per se of the UL12 protein appears to perform an important replicative function (Goldstein & Weller; 1998; Henderson et al., 1998), but the reasons for the reduced growth of mutants in non-complementing cells remain incompletely understood. Weller and colleagues reported
only a small reduction (less than 2-fold) in viral DNA synthesis, and cleavage of DNA concatemers occurred with close to wt efficiency. However, an apparent instability of DNA-containing capsids resulted in only approximately half of the cleaved genomes being recovered in the DNase-resistant (i.e. packaged) fraction. In addition, the mutants exhibited an increased accumulation of A capsids and concomitant decrease in C capsids in the nucleus of infected cells, and very few C capsids were detectable in the cytoplasm. These results led to the proposals that, in the absence of the alkaline nuclease, aberrant genomes were generated and packaged, abortive packaging events occurred frequently and that DNA-containing capsids were defective in their ability to mature into the cytoplasm (Martinez et al., 1996b; Shao et al., 1993; Weller et al., 1990).

The use of gel electrophoretic techniques subsequently demonstrated that, in cells infected with wt HSV-1, the replicative concatemers comprise branched networks containing many X- and Y-type junctions, which are present as often as once per genome (Severini et al., 1994, 1996; Zhang et al., 1994). These structures almost certainly reflect the high frequency of recombination known to occur in HSV-1-infected cells (Brown et al., 1992), and indicate that some mechanism must exist for their resolution prior to DNA packaging. Analysis by pulsed-field gel electrophoresis (PFGE) of DNA from cells infected with a UL12 null mutant demonstrated that the replicative intermediates produced by this virus were more complex than those of wt HSV-1, and apparently contained a higher level of branched structures (Martinez et al., 1996a), suggesting a key role for the UL12 protein in their processing.

The viral alkaline nuclease has also been implicated in a second recombination-related activity. Database searches revealed that the UL12 protein is conserved throughout the herpesvirus family and homologues are widely distributed in other organisms, including certain double-stranded DNA bacteriophages and baculoviruses (Aravind et al., 2000; Bujnicki & Rychlewski, 2001; Li & Rohrmann, 2000; Mikhailov et al., 2003; Vellani & Myers, 2003). Several of the related proteins, exemplified by bacteriophage lambda exonuclease, are 5′-3′ exonucleases which, in association with a single-stranded DNA-binding protein, function as recombinases (Potente, 2001; Vellani & Myers, 2003). The UL12 protein is known to interact with the HSV-1 single-stranded DNA-binding protein UL29 (Thomas et al., 1992), and recent biochemical studies demonstrating that, together, the two proteins can mediate DNA strand exchange suggest that the UL12 protein may have a role in initiating viral recombination events (Reuven et al., 2003). The alkaline nuclease is not, however, essential for viral DNA recombination, probably due to the activity of host enzymes (Martinez et al., 1996a; Weber et al., 1988).

In order to examine further how failure to express the viral alkaline nuclease impacts on the replicative cycle, we have performed a detailed characterization of the UL12 null mutant ambUL12 (Patel et al., 1996). The impairments in its ability to replicate and package the viral genome were quantified, but these only accounted for a small proportion of the reduction in the yield of infectious progeny virus. Since egress of DNA from the nucleus did not seem to be greatly reduced, we extended our investigation to the virus particles released from cells infected with ambUL12, and now demonstrate that these are greatly reduced in their ability to initiate new rounds of infection.

**METHODS**

**Cells and viruses.** Growth and maintenance of baby hamster kidney 21 clone 13 (BHK) cells were as described previously (Stow, 2001). Vero cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 5 % fetal calf serum, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹ (EFC5). S22 cells, a complementing cell line permissive for HSV-1 UL12 null mutants (Carmichael & Weller, 1989), were propagated in EFC5 containing 300 μg G418 ml⁻¹. Human embryonal kidney (FG293) cells were maintained in DMEM supplemented with 10 % fetal calf serum, 1 % non-essential amino acids, 2 mM glutamine and antibiotics as above. The HSV-1 mutant ambUL12, which contains an amber stop codon in place of the serine codon at position 129 (Patel et al., 1996), was grown and titrated in S22 cells. Stocks of wt HSV-1 strain 17 syn+ (McGeoch et al., 1988) and the rescuant ambUL12R (hereafter referred to as 12R; Patel et al., 1996) were prepared in Vero cells.

**Analysis of DNA replication and packaging.** DNA replication and packaging assays were performed as described previously (Stow, 2001). Cells were infected with 3 p.f.u. virus per cell, incubated at 37 °C and harvested 16 h p.i. (or as stated). Total and DNase-resistant (capsidated) DNAs were prepared from duplicate samples of whole cells, and nuclear and cytoplasmic DNase-resistant DNAs were isolated from separated fractions. The DNAs were analysed by restriction enzyme digestion and Southern blot hybridization. Phosphorimages were acquired using a Personal Molecular Imager and analysed with QUANTITY ONE software (Bio-Rad).

**PFGE.** Total infected cell DNA was analysed by embedding cells in 1 % agarose blocks (CleanCut; Bio-Rad) prior to in situ lysis and proteinase K digestion as recommended by the manufacturer. DNase digestion of whole cells or nuclear and cytoplasmic fractions was performed as described previously (Stow, 2001). Reactions were terminated by addition of EDTA to a final concentration of 40 mM prior to embedding, lysis and proteinase K digestion. The blocks were washed and pieces corresponding to one-quarter of the cells from a 35 mm Petri dish were inserted into the wells of a precast 1 % agarose gel. Electrophoresis and blotting were performed as described previously (Stow, 2001).

**Preparation of extracellular virus and viral DNA.** Cell monolayers in 175 cm² tissue culture flasks were infected with 3 p.f.u. virus per cell. The medium was collected at 16 h p.i., and centrifuged at 800 g for 5 min to remove cellular debris, and then the virus particles were pelleted at 100,000 g for 30 min. Virus stocks were prepared by resuspending the pellets in 3 % of the original volume of medium. Yields of infectious virus were determined by titration on S22 cells, and particle numbers were determined by electron microscopy, using latex beads of a known concentration. To prepare DNA, the pellets of virus particles were resuspended in 0.25 ml per flask 10 mM Tris/HCl (pH 7.5), 1 mM EDTA (TE), lysed by addition of EDTA to 10 mM and SDS to 0.5 % and digested with proteinase K (1 mg ml⁻¹) for 30 min at 50 °C. The samples were then gently extracted once each with phenol and
Characterization of the HSV-1 mutant ambUL12

DNA transfections. Monolayers of S22 cells in 35 mm dishes were transfected with viral DNAs using either calcium phosphate co-precipitation in conjunction with DMSO treatment (Stow & Wilkie, 1976) or Lipofectamine with PLUS reagent (Invitrogen) as recommended by the manufacturer. The cells were incubated for 3 days at 37 °C and plaques counted.

Virus yield assay. Monolayers of Vero cells in 35 mm Petri dishes were infected with the specified viruses. One hour after addition of virus, the inoculum was removed and the monolayers were washed with ‘acid glycine’ as described previously (Stow, 2001). Incubation was continued for a further 15 h. The cells were harvested by scraping into the growth medium, sonicated and the yield of virus was determined by titration on monolayers of S22 cells.

RESULTS

DNA synthesis and packaging are impaired in ambUL12-infected cells

The ability of the UL12 null mutant ambUL12 to replicate and package its genome was compared to wt HSV-1 and a rescued virus 12R, by Southern blotting and phosphor-imager analysis. Fig. 1(a) shows representative examples of experiments performed in BHK and Vero cells. In both cell lines, the amounts of mutant DNA synthesized (total DNA samples) and stably packaged (DNase-resistant DNA samples) were lower than those observed in cells infected with wt HSV-1. Moreover, since 12R exhibited a wt phenotype, these defects resulted from the failure of ambUL12 to express a functional UL12 product.

The effects of the mutation on DNA synthesis and packaging were quantified in several repeat experiments in BHK, Vero and FG293 cells. In Vero cells, DNA synthesis and packaging were reduced 2·8 ± 2·3 and 16·5 ± 8·6 fold, respectively, compared with wt HSV-1 or 12R. Corresponding reductions of 3·2 ± 1·9 and 19·1 ± 11 fold were observed in BHK cells, and of 1·6 ± 0·5 and 15·5 ± 8·8 fold in FG293 cells. The ambUL12 mutant therefore exhibits a small decrease in DNA replication (3-fold or less) in all three cell lines, similar to that previously noted with the mutant AN-1 in Vero cells (Weller et al., 1990; Shao et al., 1993). Allowing for this decrease in DNA replication, we can conclude that there is a further reduction of at least 5-fold in the efficiency of stable ambUL12 DNA packaging in BHK, Vero and FG293 cells. In contrast, AN-1 was reported to be relatively unimpaired (less than 2-fold) in DNA packaging (Shao et al., 1993).

Single-step growth curves demonstrated that, in BHK and Vero cells, the yield of ambUL12 was consistently 200- to 1000-fold lower than wt and ambUL12R (data not shown). The above reduction in encapsidated DNA is therefore insufficient to account for this decrease in yield. Shao et al. (1993) reported that AN-1 also demonstrated a marked reduction in egress of capsids from the nucleus. Analysis of DNase-resistant DNA from the nuclear and cytoplasmic fractions of Vero and BHK cells infected with ambUL12 or wt HSV-1 showed that ambUL12 was not greatly impaired in this process, and that DNase-resistant DNA was readily detected in cytoplasmic fractions of ambUL12-infected cells (Fig. 1b). When the ratios of DNase-resistant DNA in the nuclear and cytoplasmic fractions were compared, ambUL12 was found to exhibit a small defect (within the range 1- to 3·6-fold) in nuclear egress compared with wt
HSV-1 in both Vero and BHK cells. Some variability in the proportion of packaged DNA found in the cytoplasm was observed between experiments, possibly reflecting the difficulty of obtaining clean fractions. Nevertheless, electron microscopic examination of thin sections of infected cells demonstrated the presence of cytoplasmic enveloped ambUL12 particles, albeit in reduced numbers compared with wt HSV-1 and 12R (data not shown).

**Time-course of DNA synthesis and packaging in ambUL12-infected cells**

To determine whether the loss of the UL12 function had a similar effect throughout infection, a time-course of DNA replication and packaging in BHK cells infected with ambUL12 or wt HSV-1 was performed (Fig. 2). The amounts of total and DNase-resistant DNA were quantified and the proportion of the total DNA recovered in the DNase-resistant DNA fraction was calculated for each time point. The amount of ambUL12 DNA replicated was approximately 3-fold lower than wt throughout infection, and DNA packaging was also impaired at all times examined. This result suggests that the viral alkaline nuclease has a direct or indirect involvement in both DNA replication and packaging throughout infection, including early times when relatively little DNA has been synthesized and the replication compartments are small.

**PFGE analysis of ambUL12-infected cells**

Total and packaged DNAs from BHK cells infected with ambUL12 or 12R were analysed by PFGE to determine whether the mutant virus encapsidated full-length genomes. In initial experiments, we experienced loss of packaged HSV-1 DNA when cells were embedded prior to DNase treatment and subsequent lysis, proteinase digestion and electrophoresis. This problem was circumvented by using a modified approach in which permeabilized cells or isolated nuclear and cytoplasmic fractions were incubated with DNase prior to the embedding step.

Fig. 3 shows that linear HSV genomes were detected in total cellular DNA (Fig. 3a) and DNase-treated whole cell, cytoplasmic and nuclear samples (Fig. 3b, c and d, respectively) from both ambUL12- and 12R-infected cells, whereas the material that failed to enter the gel (‘well DNA’) was degraded by DNase treatment. Quantification
revealed that, for both viruses, approximately half the linear genomes present in the total DNA sample were recovered in the whole cell packaged DNA, indicating that the stability of packaging of ambUL12 and 12R genomes and their susceptibility to DNase were similar. However, the amount of linear ambUL12 DNA present in these fractions was reduced about 20-fold compared with 12R DNA, consistent with the reduction in packaging described above. In agreement with Fig. 1(b), linear ambUL12 molecules were detected in both the nuclear and cytoplasmic fractions.

The unit-length molecules from ambUL12- and 12R-infected cells exhibited a consistent and reproducible difference in appearance irrespective of whether the samples had been digested with DNase. In contrast to the sharp band of approximately 150 kbp produced by 12R, the ambUL12 band was more diffuse, with an apparent size heterogeneity in the range 135–150 kbp. This suggests that, although ambUL12 can stably package DNA, a proportion of the molecules exhibit some form of structural abnormality.

Analysis of ambUL12 particles released from BHK and Vero cells

In view of the above results, we tested whether infection of non-permissive cells with ambUL12 might generate significant numbers of virus particles unable to initiate infection in complementing S22 cells. Since stocks of wt HSV-1 derived from extracellular virus routinely exhibit the greatest infectivity (i.e. lowest particle/p.f.u. ratios), virus progeny released from BHK or Vero cells infected with ambUL12 or 12R were titrated on monolayers of complementing S22 cells, and the numbers of released virus particles were determined by electron microscopic examination of negatively stained samples. For both viruses, the majority of these particles were enveloped. The titres, particle concentrations and calculated particle/p.f.u. ratios are shown in Table 1.

Both the total number of particles and the quantity of infectious virus were reduced in ambUL12-infected BHK or Vero cells. In the three repeat experiments in BHK cells, the yield was reduced 287- to 959-fold, whilst reduction in the number of particles was only 18- to 44-fold, resulting in considerably higher particle/p.f.u. ratios for ambUL12 than 12R. A similar effect was also seen in Vero cells. Thus, in addition to the previously noted deficiency in DNA packaging, a reduced ability of ambUL12 particles to initiate plaque formation also contributes to the overall decrease in the yield of infectious virus from BHK and Vero cells.

Characterization of DNA from ambUL12 particles

To determine whether the higher particle/p.f.u. ratios of ambUL12 progeny were a consequence of the packaging of ‘poorer quality’ genomes, we examined the DNA isolated from extracellular virus particles from infected BHK cells.

The DNA was extracted using a gentle procedure to avoid damage through ethanol precipitation and resuspension (see Methods). To determine the concentration of viral DNA, samples were cleaved with EcoRI and analysed by using agarose gels alongside known amounts of EcoRI-cleaved pGX153. Fragment EcoRI N, from near the middle of UL, was detected by hybridization to labelled pGX153 DNA, and the signal intensities for the viral and plasmid-derived bands were measured on a phosphorimager. The same known amounts of ambUL12 and 12R DNAs were then compared in gels and subsequent transfection assays.

Fig. 4(a) shows a Southern blot analysis of EcoRI and BamHI digests probed with BamHI P and BamHI K, respectively. The genome locations and fragments detected are shown in Fig. 4(c). The patterns obtained for the two DNAs with the BamHI P probe were very similar, although the ambUL12 sample appeared slightly more diffuse in the region of the gel containing the co-migrating EcoRI F and G fragments. A much greater difference was apparent when BamHI digest of the same DNAs were probed with BamHI K. Although the pattern and intensities of the bands were very similar, an additional distinct smear was present in the lane containing ambUL12 DNA.

<table>
<thead>
<tr>
<th>Yield/concentration</th>
<th>Expt 1 (Vero)</th>
<th>Expt 2 (BHK)</th>
<th>Expt 3 (BHK)</th>
<th>Expt 4 (BHK)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12R particles ml⁻¹</td>
<td>1·3 × 10¹¹</td>
<td>5·4 × 10¹¹</td>
<td>3·5 × 10¹¹</td>
<td>1·6 × 10¹¹</td>
</tr>
<tr>
<td>12R p.f.u. ml⁻¹</td>
<td>9·3 × 10⁹</td>
<td>9·2 × 10⁹</td>
<td>2·2 × 10⁹</td>
<td>7·0 × 10⁹</td>
</tr>
<tr>
<td>12R particle/p.f.u. ratio</td>
<td>14·0</td>
<td>58·7</td>
<td>159</td>
<td>22·9</td>
</tr>
<tr>
<td>ambUL12 particles ml⁻¹</td>
<td>3·6 × 10¹⁰</td>
<td>1·9 × 10¹⁰</td>
<td>8·0 × 10⁹</td>
<td>8·9 × 10⁹</td>
</tr>
<tr>
<td>ambUL12 p.f.u ml⁻¹</td>
<td>6·3 × 10⁷</td>
<td>3·2 × 10⁷</td>
<td>7·1 × 10⁶</td>
<td>7·3 × 10⁶</td>
</tr>
<tr>
<td>ambUL12 particle/p.f.u. ratio</td>
<td>571</td>
<td>594</td>
<td>1127</td>
<td>1219</td>
</tr>
</tbody>
</table>
Phosphorimager analysis showed that, whereas the total radioactivity hybridized to the two DNAs was very similar for the BamHI P probe, approximately twice as many counts hybridized to ambUL12 as to 12R DNA when BamHI K was the probe.

The hybridization pattern of the BamHI K probe with ambUL12 extracellular virion DNA was unexpected, so we determined whether intracellular DNase-resistant DNA samples, as previously examined in Figs 1 and 2, behaved similarly. The relative concentrations of ambUL12 and 12R DNA were compared, as above, by measuring the counts hybridized to the EcoRI N fragment; equivalent amounts were digested with EcoRI or BamHI and analysed as in Fig. 4(a). The phosphorimages shown in Fig. 4(b) demonstrate that an equivalent result was obtained with the DNase-resistant DNA samples, i.e. little difference was detected between the DNAs with the BamHI P probe, whereas the BamHI K probe revealed increased total hybridization and the presence of a distinct smear in the ambUL12 sample.

These results indicate that packaged ambUL12 DNA prepared by two different methods is structurally distinct from 12R DNA and that different regions of the genome appear to be differently affected. It is possible that the same structural abnormality is responsible for both the apparent size heterogeneity of ambUL12 genomes seen by PFGE (Fig. 3) and the unusual patterns of hybridization seen in Fig. 4.

Infectivity of ambUL12 DNA

Although DNA from ambUL12 particles exhibited structural abnormalities compared with 12R DNA, this did not necessarily account for the higher particle/p.f.u. ratios of the mutant virus. The infectivity of the two DNAs was therefore compared on S22 cells using both calcium phosphate- and liposome-mediated transfection procedures (Table 2).

Plaques were obtained consistently with 12R but not ambUL12 DNA. To exclude the possibility that this was due to the presence of a non-specific inhibitor of the transfection process in the ambUL12 DNA preparation, control experiments were performed in which plasmid pElacZ, which expresses E. coli β-galactosidase from a constitutive promoter (Reid et al., 2003), was co-transfected with the viral DNA. Subsequent staining of the plates for β-galactosidase expression indicated that similar numbers of positive cells were obtained in the presence of the two viral DNAs. These results therefore indicate that, in comparison with 12R, the DNA packaged into ambUL12 particles is reduced in its ability to initiate an infectious cycle.

Table 2. Infectivity of ambUL12 and 12R DNAs

DNA was prepared from ambUL12 or 12R particles released from BHK cells. Monolayers of S22 cells in 35 mm Petri dishes were transfected with 12 ng viral DNA by either calcium phosphate- or liposome-mediated procedures, and plaques were counted after 3 days. The results for three independent transfections by each procedure are shown (a, b and c).

<table>
<thead>
<tr>
<th>DNA</th>
<th>Procedure</th>
<th>a</th>
<th>b</th>
<th>c</th>
</tr>
</thead>
<tbody>
<tr>
<td>12R</td>
<td>Calcium phosphate</td>
<td>15</td>
<td>6</td>
<td>19</td>
</tr>
<tr>
<td>ambUL12</td>
<td>Calcium phosphate</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12R</td>
<td>Liposomes</td>
<td>25</td>
<td>156</td>
<td>110</td>
</tr>
<tr>
<td>ambUL12</td>
<td>Liposomes</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
**Table 3. Dominant inhibitory effect of ambUL12 DNA**

S22 cells were co-transfected using liposomes with 12 ng of each of the indicated DNAs and plaques were counted 3 days post-transfection.

<table>
<thead>
<tr>
<th>DNA(s)</th>
<th>Plaques</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>108</td>
</tr>
<tr>
<td>12R</td>
<td>305</td>
</tr>
<tr>
<td>ambUL12</td>
<td>0</td>
</tr>
<tr>
<td>wt + 12R</td>
<td>390</td>
</tr>
<tr>
<td>wt + ambUL12</td>
<td>0</td>
</tr>
</tbody>
</table>

**Dominant inhibitory effects of ambUL12 DNA and particles**

Since the particle/p.f.u. ratios for ambUL12 grown in BHK cells exceeded those of 12R by only 28- to 44-fold (Table 1), it was surprising that no ambUL12 plaques were observed under conditions when 12R produced > 100 plaques. We therefore tested whether ambUL12 might exert a dominant inhibitory effect on the infectivity of co-transfected wt HSV-1 DNA. The results of such an experiment, in which DNAs were introduced into the cells using liposomes, are shown in Table 3. The addition of ambUL12 DNA completely abolished plaque formation by wt HSV-1 DNA, whereas an approximately additive effect was observed with 12R DNA. An essentially identical result was obtained using the calcium phosphate procedure, except that fewer plaques were produced by 12R and wt HSV-1 DNAs (data not shown).

We next determined whether ambUL12 particles from BHK cells could exert a similar inhibitory effect. Monolayers of Vero cells were co-infected with the phenotypically wt virus, 12R, and ambUL12 particles from BHK cells. As a control, an ambUL12 stock prepared in complementing S22 cells (designated ambUL12-S22) was also tested as the co-infecting virus. The total virus yields were determined in S22 cells (Table 4). Marked inhibition of virus replication was seen with both 0·2 and 2·5 p.f.u. per cell of ambUL12 from BHK cells, but not with 2·5 p.f.u. per cell of ambUL12-S22. Because ambUL12 grown on BHK cells exhibits a high particle/p.f.u. ratio, it was important to determine whether this difference might be due solely to the presence of greater numbers of particles. Counts performed on the two stocks revealed a 6-fold greater particle/p.f.u. ratio for the BHK-derived virus, indicating that 2·5 p.f.u. per cell of the control virus, ambUL12-S22, represents approximately twice as many particles as 0·2 p.f.u. per cell of BHK-derived ambUL12. Therefore, like the isolated viral DNA, ambUL12 particles produced by BHK cells exert a specific inhibitory effect on the growth of wt virus.

**DISCUSSION**

The results presented in this report demonstrate that several factors contribute to the reduced yield of infectious virus when the HSV-1 UL12 null mutant ambUL12 is grown in non-complementing cells. Taken together, the observed decreases in DNA synthesis, DNA packaging and nuclear egress, coupled with an increase in the particle/p.f.u. ratio of progeny virus, can account for most or all of the reduction in virus growth. Weller and colleagues have previously examined the behaviour of UL12 null mutants in Vero cells. In comparison with their results, ambUL12 was similarly impaired in DNA synthesis, exhibited a greater defect in DNA cleavage and packaging, but was less affected in capsid egress from the nucleus into the cytoplasm (Martinez et al., 1996b; Shao et al., 1993; Weller et al., 1990). The reasons for these differences are not clear, but may reflect the use of different mutants or variations between the conditions of virus growth and cell culture in the two laboratories. Although diffusion of capsids from the nucleus during cell fractionation might tend to mask a deficiency in nuclear egress, we do not believe this to be a major factor in the case of ambUL12, since release of particles into the extracellular medium was readily detectable with both BHK and Vero cells (Table 1).

Virus particles released from non-permissive cells infected with UL12 null mutants have not previously been characterized. Our results reveal several interesting features about these particles: (i) large numbers of such particles are generated, but they exhibit higher particle/p.f.u. ratios than wt HSV-1 or a rescued virus; (ii) the genomes packaged into the particles are structurally aberrant and non-infectious in transfection assays; and (iii) both the virus particles and the DNA isolated from them can exert a dominant inhibitory effect on the replication of wt HSV-1. DNA isolated from ambUL12 particles in the cytoplasmic fraction of infected cells exhibited aberrant genomes and a lack of infectivity essentially identical to the released genomes (data not shown), indicating that no selectivity exists in the nature of particles released from the cell.

Comparisons with a rescued virus, 12R, demonstrated that...
the defects exhibited by ambUL12 were a consequence of the original mutation introduced into the UL12 gene. Interestingly, however, ambUL12 stocks derived from S22 cells exhibited significantly higher particle/p.f.u. ratios than wt HSV-1 grown on these cells, and structural abnormalities were detectable in the packaged genomes by blot hybridization (data not shown). These latter observations are consistent with previous reports that complementing cell lines, including S22, fail to support the growth of UL12 null mutants to wt levels (Shao et al., 1993; Weller et al., 1990).

The mechanistic activities of the HSV-1 alkaline nuclease during infection have not been fully elucidated, and it remains difficult to explain our observations in terms of the absence of its function. The protein has been implicated in two possible recombinational activities, namely the removal of branched structures from concatemeric viral DNA (Martinez et al., 1996a) and the initiation of single strand exchange (Reuven et al., 2003). Replicative intermediates that accumulate in the absence of the nuclease are structurally more complex than those in wt HSV-1-infected cells, and probably contain a greater frequency of branched structures (Martinez et al., 1996a). It can be envisaged that the reduction in DNA synthesis reported here, and by others (Shao et al., 1993; Martinez et al., 1996b), might result from such branches imposing topological constraints on replicating molecules and impeding the progress of the DNA synthetic machinery. An alternative explanation, that UL12 plays a role in a switch to a recombination-driven late mode of DNA synthesis, as occurs in bacteriophage T4 (Mosig, 1987), seems less likely, since the effects of the absence of the alkaline nuclease were seen as early as 6 h p.i. (Fig. 2).

The presence of a greater number of branches in replicative intermediates also provides a plausible explanation for (i) the reduced efficiency of packaging that we observed in ambUL12-infected cells, and (ii) the packaged molecules appearing to be structurally aberrant. The structural abnormalities were observed in both intracellular DNase-resistant DNA and DNA extracted from extracellular capsids, and are therefore unlikely to be artefacts of the DNA preparation. The smearing and increased hybridization to certain restriction enzyme fragments of viral DNA (Fig. 4) may reflect the presence of residual single- or double-stranded branched structures that would normally be removed by UL12 prior to encapsidation. It is interesting to note that the degree of smearing and enhanced hybridization was much greater for fragments containing the a sequence and portions of the R$_4$ and R$_5$ regions (BamHI K, Q and S) than for fragments from U$_1$, (EcoRI F, G and N). This possibly correlates with previous observations that the a sequence and repeat regions represent recombinational hot spots in the HSV-1 genome associated with segment inversion (for a review see Umene, 1999). It remains to be determined whether the presence of branched structures might also explain the diffuse migration of packaged linear ambUL12 genomes seen by PFGE (Fig. 3). Work is in progress to characterize further the structural abnormalities of the packaged ambUL12 genomes.

It is clear from our characterization of DNA from extracellular ambUL12 particles that capsids containing structurally abnormal DNA can exit from the nucleus and become enveloped. DNA transfection experiments indicated that the increased particle/p.f.u. ratios observed for the cell-released ambUL12 were likely to be primarily a consequence of the aberrant nature of the packaged DNA. Exactly why these genomes are reduced in their ability to initiate plaque formation is not known, but could reflect an incomplete gene content, or cis-acting effects that might preclude genome replication or the normal expression of one or more essential genes. The presence of multiple branches and associated strand breaks in an infecting genome, if left unrepaired, might be anticipated to impede normal movement of replication or transcription complexes, and lead to an increased frequency of double-strand breaks. The residual infectivity, on complementing S22 cells, of ambUL12 particles released from non-complementing cells possibly indicates that a small proportion of the packaged genomes contain relatively few, if any, of the abnormal structures, or that the alkaline exonuclease expressed by S22 cells can function in their repair prior to replication.

Rather surprisingly, ambUL12 DNA and particles from non-permissive cells also interfered with the infectivity of wt HSV-1. In DNA transfections, a complete inhibition of plaque formation by wt HSV-1 DNA was observed, whilst the inhibitory effect of virus particles, although dose dependent, was not as great (Tables 3 and 4). We speculate that, in cells receiving both wt and mutant viral genomes (delivered as either DNA or virus particles), recombination events might introduce aberrant structures into the wt genomes, thereby reducing their ability to give rise to infectious progeny. The effect is possibly more dramatic in the transfection experiments because the successfully transfected cells receive many more viral genomes than those infected with virus particles, thereby increasing the likelihood of recombination. Recombination between normal and aberrant genomes may also explain why UL12 null mutants tend to form tiny plaques in non-complementing cells (Martinez et al., 1996b; Shao et al., 1993; Weller et al., 1990). In this situation, the small proportion of viable progeny released from the originally infected cell might become recombinationally inactivated in the presence of an excess of abnormal genomes upon infection of surrounding cells.

Taken together, our data and previous reports (Martinez et al., 1996b; Shao et al., 1993; Weller et al., 1990) demonstrate that the failure to express a functional HSV-1 viral alkaline nuclease can have multiple effects on viral DNA synthesis and packaging, release of capsids from the nucleus, infectivity of progeny virus and structure of encapsidated DNA. Although it is tempting to conclude...
that the alkaline nuclease is a multifunctional protein, it remains possible that all these effects result from the loss of a single biochemical function such as the ability to process correctly the branched structures in replicative intermediates. Further research on the role of the alkaline nuclease in recombination events should hopefully clarify this situation.

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