Replication, recombination and packaging of amplicon DNA in cells infected with the herpes simplex virus type 1 alkaline nuclease null mutant ambUL12

Iain M. Porter† and Nigel D. Stow

MRC Virology Unit, Institute of Virology, Church Street, Glasgow G11 5JR, UK

The alkaline nuclease (AN) encoded by gene UL12 of herpes simplex virus type 1 (HSV-1) is essential for efficient virus replication but its role during the lytic cycle remains incompletely understood. Inactivation of the UL12 gene results in reductions in viral DNA synthesis, DNA packaging, egress of DNA-containing capsids from the nucleus and ability of progeny virions to initiate new cycles of infection. Mechanistically, AN has been implicated in resolving branched structures in HSV-1 replicative intermediates prior to encapsidation, and promoting DNA strand-exchange. In this study, amplicons (bacterial plasmids containing functional copies of a virus replication origin and packaging signal) were used to analyse further the defects of the UL12 null mutant ambUL12. When ambUL12 was used as a helper virus both replication and packaging of the transfected amplicon were reduced in comparison with cells infected with wild-type (wt) HSV-1, and to extents similar to those previously observed for genomic ambUL12 DNA.

By using amplicons differing at a specific restriction endonuclease site it was demonstrated that replicating molecules exhibit high frequency intermolecular recombination in both wt- and mutant-infected cells. Surprisingly, in the absence of the UL12 product, amplicons lacking a functional encapsidation signal were packaged. Moreover, these packaged molecules could be serially propagated indicating that they had been incorporated into functional virions. This difference in packaging specificity between wt HSV-1 and ambUL12 might indicate that replicative intermediates accumulating in the absence of AN contain an increased incidence of structures that can serve for the initiation of DNA packaging.

INTRODUCTION

The herpes simplex virus type 1 (HSV-1) genome is a linear double-stranded DNA that replicates in the form of high molecular mass, tandem head-to-tail concatemers. Encapsulation of the DNA involves site-specific cleavage of the concatemers into unit lengths, in a process tightly coupled to their insertion into preformed capsid structures. During replication, viral genomes recombine at high frequency giving rise to a branched network, so necessitating a mechanism for resolving such structures prior to DNA packaging (for reviews see Boehmer & Lehman, 1997; Brown et al., 2002).

Although not essential for either viral DNA synthesis or packaging, the product of the HSV-1 UL12 gene is nevertheless required for full efficiency of these processes, and in its absence yields of infectious virus are reduced by 100–1000-fold (Gao et al., 1998; Martinez et al., 1996b; Patel et al., 1996; Weller et al., 1990). The UL12 protein functions as a deoxyribonuclease with 5'-3' exonuclease and endonuclease activities, which exhibit high pH optima (Hoffmann & Cheng, 1978; Knopf & Weisshart, 1990; Strobel-Fidler & Francke, 1980), and consequently is frequently referred to as an alkaline nuclease (AN). The precise role of AN during HSV-1 infection remains incompletely understood. Phenotypic analyses of UL12 null mutants have shown that the observed decrease in virus yield results from the cumulative effect of relatively small reductions in viral DNA synthesis, DNA packaging, capsid egress from the nucleus and the ability of progeny particles to initiate new cycles of infection (Martinez et al., 1996b; Porter & Stow, 2004; Shao et al., 1993; Weller et al., 1990). In addition, analysis of replicating concatemeric DNA suggests that in the absence of AN the replicative intermediates have a more complex structure with an increased frequency of branches (Martinez et al., 1996a), whilst structural abnormalities have also been detected in the genomes of progeny virions (Porter & Stow, 2004). Taken together, these observations suggest a possible role for AN in the resolution of DNA replication intermediates.

†Present address: Division of Gene Expression and Regulation, MSI/WTB, University of Dundee, Dundee DD1 5EH, UK.
of recombination intermediates prior to DNA packaging, and this model is supported by the observation that the nuclease function, per se, of the UL12 protein is necessary for efficient replication (Goldstein & Weller, 1998b; Henderson et al., 1998). It can be envisaged that failure to correctly resolve branched structures could have an indirect impact upon DNA synthesis and the assembly of the infectious virus particle. However, attempts to demonstrate the postulated structure-specific resolvase activity in vitro were unsuccessful (Goldstein & Weller, 1998a).

Recent investigations suggest a possible role for AN in an alternative recombination-related activity. The UL12 product is conserved throughout the herpesvirus family, and proteins with recognizable homology 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 these related proteins, exemplified by bacteriophage lambda exonuclease, are subunits of recombinase enzymes comprising 5'-3' exonuclease and single-stranded DNA-binding components (Poteete, 2001; Vellani & Myers, 2003). The HSV-1 UL12 protein is known to interact with the viral single-stranded DNA-binding protein, ICP8 (Thomas et al., 1999), and biochemical assays have demonstrated that together they can mediate in vitro DNA strand-exchange, suggesting that AN may participate in initiating viral recombination events (Reuven et al., 2003, 2004). The UL12 product is not, however, essential for HSV-1 recombination, and intramolecular recombination between inverted repeats can give rise to DNA segment inversion in its absence (Martinez et al., 1996a; Weber et al., 1988).

HSV-1 amplicons, bacterial plasmids containing functional copies of a virus replication origin and packaging signal (Spaete & Frenkel, 1982), have provided a convenient approach to studying many aspects of the replication and packaging process. Transient transfection of tissue culture cells with the ampiclon, followed by superinfection with wt HSV-1 helper virus, results in the plasmid being replicated as a concatamer and packaged into virus particles. The packaged ampiclon sequences can be propagated further as defective genomes in the presence of the helper (Hodge & Stow, 2001). In this study, we have used this assay to compare the behaviour of ampiclons in cells infected with wt HSV-1 and the UL12 null mutant ambUL12. These experiments demonstrate that in the absence of UL12 intermolecular recombination can occur, and that ampiclon encapsidation displays a reduced requirement for a functional packaging signal.

METHODS

Cells and viruses. Baby hamster kidney 21 clone 13 (BHK) cells were grown in Glasgow minimal essential medium (GMEM) supplemented with 10% tryptose phosphate broth, 10% newborn calf serum, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹. After transfection or infection, they were maintained in GMEM containing 5% serum and the same antibiotics (EC5). Vero cells were maintained throughout in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% fetal calf serum, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹ (EC5). The complementing S22 cell line (Carmichael & Weller, 1989) was employed for the growth of the HSV-1 UL12 null mutant ambUL12 (Patel et al., 1996). This mutant contains an amber stop codon in place of the serine codon at position 129. The HSV-1 UL19 deletion mutant K5Δ proved on UL19-1 cells (Desai et al., 1993). Stocks of wt HSV-1 strain 17 syn- and the resistant ambUL12R (hereafter referred to as 12R; Patel et al., 1996) were prepared and titrated in BHK or Vero cells.

Plasmids. Plasmids pS1 and pSA1 (Fig. 1a) contain copies of either HSV-1 oriS alone or oriS and the Uc-DR1-Ub packaging signal, respectively, inserted into the vector pAT153 (Hodge & Stow, 2001). Plasmid pSA1x was derived by ligating the self-annealed oligonucleotide 5'-AATTCTAGA-3' into EcoRI-cleaved pSA1, thereby destroying the EcoRI site and introducing a unique XbaI site. Plasmid pE12 contains HSV-1 nt 24755-27012 (McGeoch et al., 1988), encoding full-length AN, inserted with BamHI linkers into the corresponding site of the expression vector pCMV10 (Stow et al., 1993).

Transient DNA packaging assay. Assays were performed as described by Hodge & Stow (2001). Cell monolayers in 35 mm Petri dishes were transfected by the calcium phosphate technique followed by treatment with DMSO at 4 h. Each monolayer received 0.5 ml precipitate containing the indicated plasmids (1 μg per dish, unless stated) and 12 μg calf thymus carrier DNA. At 2 h after DMSO treatment, the cells were infected with 3 p.f.u. per cell of the indicated superinfecting virus. Incubation was continued for 16 h at 37°C, the cells harvested, and total and DNase-resistant (encapsi- dated) DNA were prepared from two equal samples. DNA samples, each corresponding to the yield from one sixth of a plate, were cleaved with appropriate restriction endonucleases. The enzyme DpnI was included in all digests since it cleaves unreplicated input plasmid molecules at multiple sites but is unable to digest the unmethylated products of plasmid replication. DNA fragments were fractionated by agarose gel electrophoresis, transferred to a Hybond-N membrane (Amersham) and detected by hybridization to 32P-labelled probes. Phosphorimages were acquired using the Personal Molecular Imager and analysed with Quantity One software (Bio-Rad). Quantitated values are presented as the mean ± standard deviation.

Serial propagation of ampiclons. Medium was removed from monolayers of transfected cells at 16 h post-infection (p.i.) and plates of fresh BHK or Vero cell monolayers were inoculated with 0.5 ml supernatant supplemented with 3 p.f.u. per cell wt HSV-1. This addition of wt HSV-1 is necessary because the low yield of ambUL12 helper virus from non-complementing cells is insufficient to allow infection at a high multiplicity. After adsorption, the inoculum was removed and residual virus inactivated by an acid-glycine wash (Abbotts et al., 2000). Incubation of the duplicate plates was continued for 16 h in the absence or presence of phosphonoacetic acid (PAA; 200 μg ml⁻¹) to inhibit viral DNA synthesis. Total DNA was prepared and analysed as described above.

RESULTS

Replication and packaging of pSA1 in cells infected with ambUL12

Previous experiments had indicated that replication and packaging of ambUL12 DNA were reduced approximately 3- and 18-fold, respectively, compared with wt HSV-1 in both BHK and Vero cells (Porter & Stow, 2004). To
determine whether ambUL12 was similarly impaired in replicating and packaging an amplicon, BHK cells were transfected with pSA1 and superinfected with wt HSV-1 or ambUL12. Total and DNase-resistant DNA fractions were prepared and analysed by restriction enzyme digestion and hybridization to the parental plasmid vector, pAT153.

The structures of pSA1, replicated concatemers and packaged molecules are illustrated in Fig. 1(a) and (b). The enzymes EcoRI, SalI and PstI each cleave the long DNA concatemers into 4·3 kbp molecules corresponding in length to linearized pSA1. In addition to unit length molecules, packaged DNA also yields smaller terminal fragments whose sizes depend on the position of the cleavage/packaging signal relative to the restriction enzyme site. Thus, although terminal fragments are not resolved following EcoRI digestion, fragments of 3·1 and 3·5 kbp representing the opposite ends of packaged amplicons are readily detected following digestion with SalI and PstI, respectively.

Fig. 1(c) shows an analysis of DNA samples cleaved with EcoRI and DpnI. Bands of 4·3 kbp representing DpnI-resistant replicated pSA1 molecules were readily detected in both the total and packaged DNA samples from cells infected with either wt HSV-1 or ambUL12. Moreover, ambUL12 shows a similar deficiency in replicating and packaging pSA1 as it does its own genome. Over a large number of experiments DNA replication and packaging were reduced 5·27 ± 1·83- and 15·59 ± 7·37-fold, respectively, in the presence of ambUL12 compared with wt HSV-1.

When pE12, which constitutively expresses full-length AN

**Fig. 1.** Replication and packaging of amplicon pSA1. (a) Structure of pSA1. The ori5 and Uc-DR1-Ub containing fragments are shown as thickened lines with the site of cleavage of concatemers indicated by an arrow. E, H, B, P and S indicate the position of EcoRI, HindIII, BamHI, PstI and SalI restriction endonuclease sites, respectively. (b) The upper line illustrates the structure of a concatemer generated by pSA1 replication (only two complete copies of the monomeric plasmid are depicted). Concatemers are cleaved within the packaging signals (arrows), generating packaged molecules with Uc and Ub at opposite ends, which yield diagnostic terminal fragments. The sizes of the large PstI (P) and SalI (S) terminal fragments are shown in kbp. (c) BHK cells were transfected with pSA1 in the presence or absence of 1 μg per plate pE12 and superinfected with wt HSV-1 (wt) or ambUL12 as indicated. After 16 h, the cells were harvested and total and DNase-resistant DNA prepared as described in Methods. DNA samples were cleaved with EcoRI plus DpnI, fractionated by agarose gel electrophoresis, blotted and hybridized to 32P-labelled pAT153 DNA. After washing, the membrane was exposed to a phosphorimager screen for either 2 h (left-hand panel) or 16 h (right-hand panel). The position of linear pSA1 molecules (4·3 kbp) is indicated. (d) Samples of packaged DNA from cells transfected with pSA1 and superinfected with wt HSV-1 (wt) or ambUL12 (amb) were cleaved with either SalI or PstI (in combination with DpnI) and analysed as in (c). The positions and sizes of monomers and the larger terminal fragment are indicated for each enzyme.
under the control of the human cytomegalovirus (HCMV) major immediate early promoter, was co-transfected with pSA1, small increases in replication and packaging were observed in the presence of ambUL12. Analysis of five independent experiments revealed 2-24 ± 0-40- and 2-54 ± 1-06-fold increases in replication and packaging, respectively, in cells that also received pE12. Co-transfection of the vector pCMV10 had no significant effect on replication or packaging, and neither pE12 nor pCMV10 had any effect in cells infected with wt HSV-1 (data not shown). The behaviour of the rescued virus 12R was indistinguishable from wt HSV-1, and very similar impairments in pSA1 replication and packaging were observed in Vero cells (examples shown later in Figs 3 and 4). These results demonstrate that failure to express the UL12 protein reduces both replication and packaging of the amplicon pSA1, and that the defect of the null mutant can be overcome to a small extent by co-transfection of pE12.

When DNase-resistant DNA was cleaved with SalI or PstI, the large fragments originating from the opposite ends of the packaged molecules were readily detected in samples from both wt HSV-1- and ambUL12-infected cells (Fig. 1d). Thus, cleavage events within the Uc-DR1-Ub packaging signal to initiate and terminate packaging do not require the viral AN.

**Intermolecular recombination between replicated amplicons**

In order to test for intermolecular recombination between concatamers, a derivative of pSA1, pSA1x, in which the unique EcoRI site had been converted to a unique XbaI site, was constructed. BHK cells were transfected with pSA1 and pSA1x alone or in combination and superinfected with wt HSV-1 or ambUL12. Total DNA was prepared, digested with EcoRI, XbaI or both enzymes together. The digestion products were fractionated and hybridized to 32P-labelled pAT153 DNA (Fig. 2).

In cells transfected with pSA1 or pSA1x alone and superinfected with ambUL12, high molecular mass concatamers were generated which, as expected, were cleaved into monomeric units by EcoRI or XbaI, respectively, but were resistant to the other enzyme. The concatamers generated in cells that received both plasmids were cleaved into monomers by a combination of EcoRI and XbaI. Digestion with EcoRI alone yielded fragments that co-migrated with the concatamers and monomers produced in individually transfected cells, but also a ladder of fragments of intermediate size. This demonstrates that individual concatamers contain both EcoRI and XbaI sites and is indicative of high frequency recombination between the products of replication of the two input plasmids. Similar generation of a ladder of bands was observed with EcoRI-cleaved DNA from doubly transfected cells superinfected with wt HSV-1. The relative molar ratios of the well-resolved monomer, dimer and trimer bands were measured at 100:7:7:7:5 and 100:5:4:4:2 in the ambUL12 and wt HSV-1 samples, respectively. These data indicate that the viral AN is not required for the observed intermolecular recombination events.

**Packaging of DNA lacking an encapsidation signal by ambUL12**

Plasmid pS1 is identical to the amplicon pSA1 but lacks the Uc-DR1-Ub packaging signal. Surprisingly, preliminary experiments using this plasmid as a control indicated that pS1 was not only replicated but also encapsidated when ambUL12 was employed as superinfecting virus. The relative abilities of wt HSV-1 and ambUL12 to replicate and package pS1 were therefore investigated in detail.

BHK cells were transfected with pSA1, pS1 or pAT153 (which lacks both oriS and a packaging signal) and superinfected with ambUL12 or wt HSV-1 (wt). Total DNA was prepared and cleaved with EcoRI (E), XbaI (X) or both (EX) in the additional presence of DpnI. Fragments were analysed as described in Fig. 1. The position of the 4-3 kbp plasmid monomer band is indicated. The left-hand panel (ambUL12 samples) represents an approximately 2.5 times longer exposure to a phosphorimager screen than the right-hand panel (wt samples).
Total and DNase-resistant DNAs were cleaved with EcoRI and DpnI and analysed as before (Fig. 3a). Plasmids pSA1 and pS1 were replicated with similar efficiency in the presence of wt HSV-1 (total DNA samples), but only pSA1 was packaged to a significant level (DNase-resistant DNA fractions). A very faint band of DNase-resistant pS1 DNA was observed but this represented <0.5% of the corresponding pSA1 signal. K5A2 replicated the two plasmids to similar extents to wt HSV-1, packaging to an extent only slightly less than pSA1, and significantly greater than when wt HSV-1 was helper. Also, when AN was co-expressed from plasmid pE12, the ability of ambUL12 to replicate pS1 was enhanced (as previously noted for pSA1 in Fig. 1) but the amount of encapsidated product was reduced. These results demonstrate that failure to express a functional UL12 product is associated with a significantly increased ability to package an amplicon lacking an HSV-1 packaging signal.

A similar experiment was performed in Vero cells but the EcoRI and DpnI digested DNAs were hybridized to a 32P-labelled plasmid (pGX153) comprising the HSV-1 BamHI p fragment inserted into pAT153. This probe allows simultaneous detection of the helper virus EcoRI N fragment (2.4 kbp) and the replicated amplicon (4.3 kbp). A very similar result was obtained (Fig. 3b). Packaging of pS1 was detected in cells infected with ambUL12 but not wt HSV-1, and pE12 again enhanced replication but reduced packaging of pS1 in the presence of the mutant. The increased packaging of replicated pS1 molecules in the absence of the viral AN is therefore not a cell-specific phenomenon associated with BHK cells.

Quantitative analysis of repeat experiments indicated that the amounts of pS1 packaged in ambUL12-infected BHK and Vero cells were 27.6 ± 10.2 and 31.4 ± 16.6%, respectively, of the amount of pSA1 packaged. In contrast, the pS1 signal was consistently <0.5% of that for pSA1 in wt HSV-1-infected BHK cells, and below the level of detection in Vero cells.

**Propagation of packaged pS1 molecules**

Since pS1 molecules were packaged in cells infected with ambUL12, it was of interest to determine whether the particles containing the plasmid sequences matured into virions capable of infecting fresh cells. Transient assays were set up in which BHK cells were transfected with pS1 or pSA1 and superinfected with wt HSV-1, ambUL12 or the resent 12R. As before, total and DNase-resistant DNA fractions were prepared from the transfected cells at 16 h p.i., but in this instance the supernatant media were also removed and retained. Samples of these supernatants were used to infect fresh monolayers of BHK cells to test whether the amplicons could be serially propagated. However, since only very low yields of infectious ambUL12 are released from infected BHK cells (Porter & Stow, 2004), it was necessary to add the equivalent of 3 p.f.u. per cell wt HSV-1 to each inoculum in order to ensure the expression of the necessary helper functions for replication of the defective genomes. To confirm that any hybridization to plasmid vector sequences represented de novo replication, infections were performed in duplicate, the plates were acid-glycine washed after adsorption to remove residual virus, and one of each pair was incubated in the presence of 200 μg PAA ml⁻¹ to inhibit viral DNA synthesis. Total DNA was prepared from all plates at 16 h p.i.

DNA samples were cleaved with EcoRI plus DpnI, fractionated and hybridized to 32P-labelled pAT153. The results are
shown in Fig. 4. The patterns of replication and packaging seen with the DNA samples isolated from the cells transfected with pS1 or pSA1 and superinfected with wt HSV-1 or ambUL12 (Fig. 4a) were very similar to those observed before (Fig. 3a). In addition, the behaviour of the rescued virus 12R was indistinguishable from wt HSV-1 confirming that the altered properties of ambUL12, and in particular its relatively enhanced packaging of pS1, are consequences of the lesion in the UL12 gene.

Fig. 4(b) shows the result of serial propagation of the supernatant medium in the presence or absence of PAA. Plasmid sequences were undetectable in the DNA from PAA treated plates, demonstrating that residual DNA from the inocula was not responsible for the hybridization signal seen with untreated plates. As previously shown (Hodge & Stow, 2001), pSA1 but not pS1 could be serially propagated from cells superinfected with wt HSV-1, and the same result was obtained when 12R was the superinfecting virus. In contrast, both pS1 and pSA1 were serially propagated from the supernatant media of the cells originally superinfected with ambUL12. In a separate experiment (data not shown), it was found that serial propagation of both plasmids also occurred when the supernatant from cells initially infected with ambUL12 was supplemented with 3 p.f.u. per cell ambUL12 in place of wt HSV-1. However, as also seen in transfected cells, reduced levels of plasmid sequences accumulated in the absence of AN expression by the helper virus.

Due to the absence of a packaging signal, monomeric pS1 is approximately 200 bp smaller than pSA1 and therefore migrates slightly faster. This difference, which is apparent in all the DNA samples from transfected cells (Figs 3 and 4a), was retained in the propagated amplicons from the ambUL12-infected cells. These data indicate that in the absence of the viral AN, packaged pS1 molecules are released from cells in a form (presumably as virions) capable of delivery to previously uninfected cells; and that the expression of helper functions allows their subsequent replication without major structural alteration. The ability of pS1 to be packaged and propagated is therefore unlikely to be a consequence of the acquisition of a packaging signal through recombination with the helper virus.
Packaged pS1 molecules are high molecular mass concatamers

Although EcoRI digestion of DNA samples from cells transfected with pS1 and superinfected with ambUL12 yielded fragments the size of linear plasmid, it remained possible that the ability of these sequences to be packaged and propagated arose from their integration into the viral genome. To exclude this possibility, packaged DNAs were also analysed following treatment with DpnI alone or KpnI plus DpnI. The enzyme KpnI cleaves HSV-1 DNA 26 times, generating a largest fragment of 12-8 kbp. DpnI was included to cleave any unreplicated input plasmid molecules.

Blots were hybridized to 32P-labelled pAT153 or HSV-1 DNA to detect plasmid and viral fragments, respectively, and the results are shown in Fig. 5. The patterns of fragments hybridizing to the pAT153 probe (Fig. 5a) were essentially unaltered following digestion with KpnI. Consistent with previous results, pSA1 replicated and packaged in the presence of wt HSV-1 consisted of a ladder of bands representing the packaging of concatameric molecules consisting of integral numbers of copies of the monomeric unit up to the length of a standard HSV-1 genome (in this particular gel, only fragments up to approximately 30 kbp, corresponding to a 7-mer, were resolved). A similar ladder was observed with pSA1 when ambUL12 was the superinfecting virus. Plasmid pS1 packaged in the presence of ambUL12 ran predominantly as a high molecular mass band (> 30 kbp) resistant to KpnI cleavage, but no ladder was discernible, even upon longer exposure or manipulation of the phosphorimage. Were pS1 to be integrating into the viral genome, an insertion in excess of 17 kbp into even the largest KpnI fragment would be required to generate a fragment with this mobility. This seems unlikely since indirect evidence suggests that packaging efficiency decreases rapidly as genome length approaches 170 kbp (Saeki et al., 2001). Moreover, KpnI fragments >30 kbp were not detected with the HSV-1 probe (Fig. 5b). These results therefore strongly suggest that, like pSA1, pS1 is replicated and packaged as an independent entity in ambUL12-infected cells.

DISCUSSION

The previously observed deficiencies in genome replication and packaging exhibited by UL12 null mutants (Martinez et al., 1996b; Porter & Stow, 2004; Shao et al., 1993; Weller et al., 1990) were faithfully recapitulated in a transient assay using the amplicon pSA1. This assay, in turn, provided a means of investigating intermolecular recombination and the requirements for the cis-acting viral packaging signal in the absence of the UL12 product. The major findings were that intermolecular recombination between replicated amplicons occurred efficiently in cells infected with either wt HSV-1 or the UL12 null mutant ambUL12, and that there was a reduced requirement for the viral packaging signal in mutant-infected cells.

Although the use of the rescuant 12R demonstrated that the altered properties of ambUL12 were a result of the UL12 lesion, only very inefficient complementation of pSA1 replication and packaging were observed upon cotransfection of a plasmid, pE12, expressing the full-length protein. There appear to be exacting requirements for full complementation of UL12 null mutants by UL12 expressed in trans, and even the available cell lines expressing UL12 do not support the growth of the mutant viruses to wt levels (Shao et al., 1993; Weller et al., 1990). The amounts of UL12 expressed per cell were similar for cells transfected with pE12 or infected with wt HSV-1 (data not shown), but it is not known whether the timing of expression or processing were the same in both cases. In our transient transfection assays it is also possible that many of the cells undergoing pSA1 replication and packaging were not expressing the UL12 protein.

A previous study demonstrated that viral DNA recombination can occur in the absence of AN, but examined intramolecular inversion events between inverted repeats, either in the viral genome or specifically constructed amplicons (Martinez et al., 1996a). In the light of the finding that AN and ICP8 can promote DNA strand-exchange together (Reuven et al., 2003, 2004), it was interesting to determine whether the absence of the UL12 product might affect

![Fig. 5. Analysis of packaged pS1 DNA. Packaged DNA from BHK cells transfected with pS1 or pSA1 and superinfected with wt HSV-1 or ambUL12 (amb), as indicated, was cleaved with DpnI alone (D) or in combination with KpnI (K+D). The digested DNAs were fractionated on a 0.6% agarose gel, blotted and hybridized to 32P-labelled pAT153 (a) or HSV-1 (b) DNA. The position of genome length DNA is indicated by an arrowhead, and the sizes of selected other bands (kbp) are shown.](http://vir.sgmjournals.org)
intermolecular recombination. Our demonstration of intermolecular recombination between amplicons replicated in the presence of wt HSV-1 (Fig. 2) agrees with the findings of Fu et al. (2002). A very similar pattern of recombination products was observed in cells infected with ambUL12, demonstrating that the recombinase activity of the viral AN is also non-essential for intermolecular recombination events. It is likely that host enzymes, perhaps in conjunction with the HSV-1 single-stranded DNA-binding protein ICP8, mediate the observed recombination. In this context, it is interesting to note that ICP8 has been demonstrated to mediate strand-invasion and -exchange in the absence of AN, and also to interact with host proteins known to be involved in DNA replication and repair (Nimonkar & Boehmer, 2002, 2003; Taylor & Knipe, 2004).

The enhanced encapsidation of an amplicon lacking viral packaging signals in cells infected with ambUL12 was unexpected. Packaged and propagated pS1 molecules were unaltered in size (Fig. 4), but in contrast to pS1, the packaged pS1 molecules did not generate a discernible ladder (Fig. 5), or yield distinct terminal fragments when digested with various enzymes (data not shown). It therefore appears unlikely that a packaging signal has been acquired by recombination with the helper virus or that a single alternative sequence in pS1 is recognized for cleavage and packaging. Rather, the ends of the packaged molecules appear to be randomly distributed.

By analogy to bacteriophage systems, it is believed that a complex of the HSV-1 UL15 and UL28 proteins functions as a terminase responsible for site-specific cleavage of concatemeric DNA and the coupled insertion of unit length genomes into the capsid (Brown et al., 2002). The UL28 component is directly involved in recognition of the cleavage-packaging signal (Adelman et al., 2001), and a similar role has been demonstrated for the homologous protein of HCMV (Bogner et al., 1998). Viral AN is non-essential for the site-specific cleavage of genomic concatemers (Martinez et al., 1996a; Porter & Stow, 2004) and, in agreement, similar specific terminal fragments of packaged pSA1 DNA were detected in both ambUL12 and wt HSV-1-infected cells (Fig. 1d). Nevertheless, it remains possible that the presence of the UL12 product is important for maintaining full sequence specificity of the terminase, and that in its absence cleavage of concatemers at alternative sites can occur to initiate and terminate packaging. No direct physical association of the UL12 protein with either UL15 or UL28 was detected in immunoprecipitation experiments with extracts from insect cells infected with baculovirus expression vectors (I. Porter, unpublished results). This suggests that any alteration in cleavage specificity is more likely to occur by an indirect mechanism. The studies of Adelman et al. (2001) indicated that the secondary structure of the packaging signal was important for UL28 recognition. Perhaps similar structures, also recognizable by UL28, are generated elsewhere in the genome during replication and recombination, and the nucleolytic activity of the UL12 protein participates in their removal.

During DNA replication events such as the stalling of replication forks can lead to the generation of double-strand breaks. Recombination is essential for the repair of such lesions and the maintenance of genome integrity. It can be envisaged that the increased complexity of branched concatemers accumulating in the absence of AN (Martinez et al., 1996a) might lead to a higher frequency of double-strand breaks. Alternatively (or perhaps additionally), AN may play a role in their repair, ensuring that these lesions are normally short lived and do not accumulate in concatemeric DNA. In either case, viral DNA replicated in the absence of AN would be expected to contain a greater number of free DNA ends. Although it is free ends generated by the terminase activity that normally serve for the initiation of packaging, it remains possible that other free ends in concatemeric viral DNA might function similarly. Direct evidence for an increased incidence of double-strand breaks in the absence of UL12, or for initiation of packaging at randomly generated ends, is lacking, but such mechanisms would provide an alternative explanation for the observed packaging of pS1 DNA in ambUL12-infected cells.

Several interesting points relate to the ability of the packaged pS1 molecules to be serially propagated. This observation indicates that, irrespective of the mechanism of packaging, the maturation of DNA containing capsids into infectious extracellular virions can proceed as normal. In addition, virion DNA lacking a functional packaging signal must be able to enter the nucleus, reach the appropriate site and adopt the required configuration for DNA replication. The standard model, in which circularization of input HSV-1 genomes is required for viral DNA synthesis (Boehmer & Lehman, 1997), has recently been challenged (Jackson & DeLuca, 2003) and this issue remains to be resolved. Nevertheless, it should be noted that packaged molecules containing multiple tandem copies of pS1 are likely to be capable of efficient circularization through homologous recombination, even though the non-specific nature of the cleavage events may result in their termini being incapable of direct ligation.

The ability of amplicons that lack a packaging signal to be encapsidated in the absence of the UL12 product may therefore provide a useful approach not only for further functional analysis of the viral AN, but also for investigating other aspects of the lytic cycle including the replication and maturation of viral DNA and the behaviour of infecting genomes.

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