Intracellular Cre-Mediated Deletion of the Unique Packaging Signal Carried by a Herpes Simplex Virus Type 1 Recombinant and Its Relationship to the Cleavage-Packaging Process

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To gain further insight on the function of the herpes simplex virus type 1 (HSV-1) packaging signal (a sequence), we constructed a recombinant virus containing a unique a sequence, which was flanked by two loxP sites in parallel orientation. The phenotype of this recombinant, named HSV-1 LaL, was studied in cell lines which either express or do not express Cre recombinase. Although LaL virus multiplication was only slightly reduced in standard cell lines, its growth was strongly inhibited in Cre-expressing cells. In these cells, a sequences were detected mostly in low-molecular-weight DNA circles, indicating that they had been excised from virus DNA by site-specific recombination. Deletion of the a sequences from the viral genome resulted in the accumulation of uncleaved replication intermediates, as observed by pulsed-field gel electrophoresis. B-type capsids also accumulated in these cells, as shown both by electron microscopy and by sucrose gradient sedimentation. Further examination of the status of a sequences in Cre-expressing cells indicated that high-level amplification of this sequence can occur in the absence of the cleavage-packaging process. Moreover, the amplified a signals in small circular DNA molecules remained uncleaved, indicating that these molecules were not able to efficiently interact with the cleavage-packaging machinery. The cleavage-packaging machinery and the structural proteins required to assemble virions were, however, functional in HSV-1 LaL-infected Cre-expressing cells, since this system could be used to package plasmid DNA harboring an origin of virus replication and one normal a signal. This is the first study in which accumulation both of uncleaved replication intermediates and of B capsids has been obtained in the presence of the full set of proteins required to package virus DNA.

The genome of herpes simplex virus type 1 (HSV-1) is a linear double-stranded DNA molecule of 152 kbp, consisting of two covalently linked components, L and S, that constitute, respectively, 82 and 18% of the genome. The L and the S components are composed of unique long (UL) and unique short (US) sequences flanked by pairs of inverted repeat sequences, known as the b (9 kbp) and c (6.5 kbp) sequences, respectively. The genome is also flanked at each end by a direct repeat known as the a sequence, and inverted copies of this sequence separate the L and S components. Variable numbers of a sequences can be present both at the L terminus and at the L-S junction, but a single a sequence is generally present at the S terminus (5, 19). The standard virus genome can thus be represented by a,b-ULa,b a,b ULa,b a,b ULa,b with n and m varying from 1 to more than 10. The L and S components can invert relative to each other, generating four isomeric forms of viral DNA.

The a sequence, which varies in size from 280 to 550 bp among HSV-1 strains, contains unique (U) and directly repeated (DR) sequence elements. As an example, the a sequence of HSV-1 (F) has the structure (DR1)-Uc-(DR4),- (DR2),-Ub-DR1, where DR1, DR2, and DR4 are 20, 12, and 37 bp long, respectively, and Uc and Ub are 58 and 64 bp long, respectively (18). DR2 varies from 19 to 22 copies per HSV-1 (F) a sequence, whereas DR4 varies from 2 to 3 copies per a sequence. Tandemly reiterated a sequences share the intervening DR1. Free L terminus and S terminus each contain only a portion of the 20-bp DR1 sequence and together form one complete DR1 sequence (19).

The a sequence is an important cis-acting sequence in the HSV-1 replication cycle. It is the site of end joining (circularization) of the genome soon after infection (20, 25). In addition, this sequence carries the signals for the two separate cleavage events required to generate mature, packaged genomes from the replication concatemers (6, 33). The cleavage event has been related to amplification of the a sequence, inasmuch as molecules carrying cab junctions are cleaved to generate ab and ca termini (5, 33). However, recent data suggest that amplification of a signals could also be an early event that occurs before the cleavage of the concatemers (2). Lastly, cleavage of concatameric intermediates seems to occur in a directional manner, since only L ends have been observed in such replication intermediates (1, 2, 17, 31).

Cleavage of the a sequence appears to be tightly linked with packaging. First, in infected cells the mature linear unit-length genomes can be recovered only as packaged DNA. Second, most mutants with altered cleavage-packaging machinery accumulate uncleaved replication concatemers (13, 26, 29, 36). To date, only a mutant with the UL25 gene product affected (KUL25NS) presents the particular phenotype of a cleaved DNA in the absence of packaging, indicating that this protein is essential for retaining DNA in capsids (16). Last, cleavage and packaging of the viral genome is biologically linked with capsid formation since HSV-1 mutants that fail to make capsids due to deletions of genes encoding essential capsid proteins (such as UL18 or UL19) are found to synthesize wild-type levels of virus DNA but fail to cleave concatemer DNA into genomic units (7).

Three types of capsids accumulate in the nuclei of HSV-1-
infected cells, and they are designated A, B, and C, according to their sedimentation position through sucrose gradients (10). B-type capsids contain an electron translucent core of scaffolding protein. C-type capsids contain the viral DNA genome instead of this core and are thought to be the precursors of enveloped virions. A-type capsids are empty capsids thought to result from abortive attempts to package DNA. In agreement with this last idea, mutant KUL25NS shows an abnormally abundant amount of A capsids (16).

Most of our knowledge about cleavage-packaging of viral DNA in HSV is derived through the use of viral mutants containing individual deletions of each of the concerned proteins. In each of these cases, however, the absence of a particular trans-acting function hampers the molecular analysis of this cleavage-packaging. To gain further insight into the role and function of the HSV-1 σ sequence during HSV-1 replication, in the presence of the full set of trans-acting functions, we set out to delete in situ this essential cis-acting sequence in infected cells. For this, we took advantage of previous works showing that Cre-loxP site-specific recombination can be used to intracellularly manipulate HSV-1 virus and vector genomes (14) and to delete the packaging signal of adenovirus (24). We have thus constructed a recombinant HSV-1 virus, named HSV-1 LaL, carrying a unique packaging signal which, in addition, is bracketed by loxP sites in the same orientation, and we have studied the phenotype of this recombinant in control cells and in cells expressing Cre recombinase.

MATERIALS AND METHODS

Cells. Vero, TE-671 (human rhabdomysarcoma cells, ATCC CRL 8805), and TE-CRE30 (14) cell lines were propagated in Dulbecco’s minimum essential medium (Biomedica) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL), penicillin (100 U/ml) and streptomycin (100 mg/ml). BHK-21 cells were propagated in Dulbecco’s minimum essential medium supplemented with 10% FBS, 10% tryptose-phosphate broth, and antibiotics. Wild-type KOS was used in this study. Virus production and titration were carried out as previously described (3).

Construction of pA-LaL. Plasmid pA-LaL (Fig. 1A) is identical to the previously described pA-ZeoSVLacZ (14), except for the fact that its cleavage-packaging signal is bracketed by loxP sites (“floxed”). To create this plasmid, a 2.5-kb fragment containing the σ sequence was excised from pA-ZeoSVLacZ by EcoRI digestion. The digested plasmid was religated, creating pA-ZeoSVlacZa. The ends of the 1.2-kb EcoRI fragment were made blunt and inserted into the Smal site of pBS246 (Gibco BRL) in order to place the a sequence between the two head-to-tail loxP sites, creating plasmid pLaL. Then, a 1.5-kb NotI fragment, containing the floxed σ sequence, was cleaved from pLaL, made blunt ended, and inserted into the blunt-ended unique EcoRI site of pA-ZeoSVlacZa. Enzymes and molecular weight standards were purchased from Biolabs and Gibco BRL and used according to the manufacturers’ recommendations. Plasmids were selected and produced in Subcloning Efficiency DH5α competent cells (Gibco BRL).

Construction of cos56LaL. To construct cos56LaL, we used the floxed σ sequence of plasmid pLaL. The 1.5-kb NotI fragment, containing the floxed σ sequence, was cleaved from pLaL, made blunt ended, and inserted into the unique blunted-end XbaI site of cos6 (4) to create cos56LaL, using a GigapackIII kit (Stratagene).

Production of ampiclon vectors A-LaL. Five micrograms of ampiclon plasmid pA-LaL was transfected into TE-671 cells using the calcium phosphate procedure (11). Transfected cells were superinfected the next day with HSV-1 KOS at a multiplicity of infection (MOI) of 0.1 PFU/cell in medium 199 (Gibco BRL) supplemented with 1% FBS. When total cytopathic effect was observed, cells were frozen and thawed three times in liquid nitrogen to release the infectious virus. When required, ampiclon stocks were serially passaged onto fresh TE-671 cells, by using a 1:2 dilution, as previously described (32). Titers of helper virus were determined by plaque assay on Vero cells. In the case of ampiclon vectors expressing f-galactosidase, vector titers were assessed by scoring the number of blue infected cells following fixation and 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal) staining. The ampiclon stocks produced contained between 10^5 and 10^6 PFU of helper virus particles/ml and generally had helper-to-vector ratios of about 1:10.

Construction of LaL virus. To create HSV-1 LaL, we used the cosmid set covering the entire HSV-1 sequence except the σ sequences (4, 8). Cosmids cos6, cos14, cos28, and cos56 were labeled with [α-32P]dCTP (ICN Biomedicals) as instructed by the manufacturer. After separation on an agarose gel, DNA was UV depurinated and the gel was denatured, neutralized, and transferred to filters (Hybond N+, Amersham) using a vacuum blotting system (Pharmacia), as previously described. Filters were then prehybridized and hybridized at 65°C for 3 h, as already described (3). Filters were then washed and exposed to autoradiographic film ( Kodak) at −70°C.

Transmission electron microscopy. Cells were infected at 0.5 PFU/cell. At 24 h postinfection, cells were washed with PBS and fixed for 3 h at room temperature with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Cells were then rinsed several times and stored at 4°C in 0.15 M cacodylate buffer, pH 7.4, before being processed into thin sections.

Purification of virus capsids. Five 152-ml flasks of confluent TE-671 or TE-CRE30 cells were infected with HSV-1 LaL at a MOI of 0.1 PFU/cell. At 24 h postinfection, cells were collected by centrifugation at 4,000 rpm in a GSA rotor for 10 min, rinsed with PBS, and resuspended in lysis buffer (1% Triton X-100, 0.5 M NaCl, 1 mM EDTA, 20 mM Tris (pH 7.6), 1 mM dithiothreitol, 1 mM EDTA). Cells were then digested by proteinase K and phenol-chloroform extraction. DNA was obtained through the use of viral mutants containing a unique packaging signal which, in addition, is bracketed by loxP sites in the same orientation, and we have studied the phenotype of this recombinant in control cells and in cells expressing Cre recombinase.
FIG. 1. Site-specific deletion of amplicon vector A-LaL packaging signal. (A) Structure of pA-LaL amplicon plasmid used to generate the corresponding A-LaL vector, using KOS virus as helper in TE-671 cells. (B) Expected products of partial and total Cre-induced specific recombination at the loxP sites carried by amplicon vector DNA. These are concatemers that have lost most a sequences and loxP sites and plasmids containing one or two loxP sites (circles). Small circular nonplasmidic molecules, products of Cre-loxP-mediated deletion, containing just one a sequence and a loxP site, are also shown (ovals). (C) MluI-restricted plasmid DNA extracted from Escherichia coli cells that had been transformed with LMW DNA extracted from A-LaL-infected TE-CRE30 cells. The parent pA-LaL plasmid contains four MluI sites, which gives rise to fragments of 3,917, 3,813, 780, and 425 bp. LMW DNA extracted from TE-CRE30 cells had lost the 3.8-kbp fragment. Instead, a 2.4-kbp fragment, corresponding to the same fragment after deletion of the a signal and a loxP site (pA-LaLΔaΔloxP), was apparent. (D) Two different A-LaL vector populations, generated using wild-type virus as helper, were serially passaged on TE-671 cells (light gray) or on TE-CRE30 cells (hatched). An aliquot of the virus population that had been passaged once in TE-671 cells was further passaged twice on TE-CRE30 cells (dark gray). In all cases, after each passage, vector and helper particles were independently titrated on Vero cells, and the evolution of vector-to-helper ratios during three successive passages is presented.
RESULTS

Use of Cre-loxP recombination system to inhibit packaging of amplicon vector genome. We and others have previously shown that the Cre-loxP recombination system can be used to manipulate HSV-1 and helper-dependent amplicon vectors (14, 27). These vectors derive from a plasmid (the amplicon plasmid) which carries one origin of virus replication (ori-S) and one packaging signal (a) (5, 19, 20, 32, 34). As a preliminary step of the present study, we studied whether Cre-loxP recombination could be used to delete floxed a cis-acting sequence from an amplicon vector genome. Our goal was to use this convenient and simple model to determine (i) if a floxed sequence is still recognized by the cleavage-packaging machinery and (ii) if deletion of the sequence is still recognized by the cleavage-packaging machinery. We next studied the efficiency of intracellular Cre-mediated recombination on such a vector, using TE-CRE30 cells, a Cre-expressing cell line previously described (14). Since the concatemeric DNA generated from pA-LaL contains two loxP sites per genomic monomer, a complex pattern of Cre-mediated recombination is expected to occur, and some examples are shown in Fig. 1B. Theoretically, if all loxP sites carried by these molecules were involved in recombination (either in one or in successive waves of recombination), the final product should be a pA-LaL plasmid with a loxP-a sequence deleted. Intermediate products of recombination, like concatemers and plasmids with a sequence deleted and harboring more than one loxP site were also expected to occur. We then designed experiments to analyze the structure of plasmids regenerated by site-specific recombination, as follows. The floxed A-LaL vector virus was used to infect TE-671 and TE-CRE30 cells at a low MOI. The following day, low-molecular-weight (LMW) DNAs were extracted from both cell cultures and used to transform competent bacteria, as previously described (14). We obtained 126 phleomycin-resistant and β-galactosidase-positive clones from DNA taken from TE-CRE30-infected cells versus none from TE-671-infected cells. This indicates that such LMW molecules were generated only in cells expressing Cre. Analysis of plasmid DNA isolated from several colonies demonstrated that all had lost the 3.8-kbp fragment containing the a sequence (Fig. 1C). These plasmids, designated pA-LaLΔloxP-a plasmids, contained instead a new 2.4-kbp fragment, consistent with the expected site-specific recombination product. Failure to observe intermediate plasmids containing floxed a signals suggests that recombination was rather efficient and precluded detection of intermediate forms.

To study the impact of site-specific deletion of the sequence on packaging efficiency, the fate of A-LaL amplicon vector preparations was monitored during serial passages either on TE-CRE30 or on TE-671 control cells. Both cell cultures were transfected with 5 μg of pA-LaL, and cells were then superinfected with wild-type helper virus at a MOI of 0.1. The resultant replication-competent vector stocks were then serially passaged in their respective cell lines. After each passage, both vector and helper particles were titrated on Vero cells, and the evolution of the relative fraction of vector-to-helper particles obtained during three successive passages was plotted. As shown in Fig. 1D, the fraction of vector particles serially passaged on TE-671 cells presented a classical up-and-down growth curve of defective interfering particles. In contrast, on TE-CRE30 cells, a regular decrease in the vector fraction was observed after each passage, resulting in a 2-log reduction in titer, compared to the fraction obtained in TE-671 cells. An aliquot of the vector population that had been passaged once in TE-671 cells was further passaged on TE-CRE30 cells. Again, the fraction of vector particles produced on TE-CRE30 cells steadily decreased to reach very low levels after two passages. Control experiments performed on TE-CRE30 cells, using amplicons without loxP sites, showed no steady decrease in vector fractions (data not shown). These data strongly suggest that deletion of the floxed a signals resulted in specific inhibition of vector DNA packaging. Taken together, these results indicate (i) that vector genomes carrying floxed a signals were efficiently packaged in cells expressing no Cre recombinase and (ii) that packaging of these genomes was efficiently inhibited in Cre-expressing cells. We thus applied this principle to recombinant HSV-1, by introducing the floxed a sequence from pA-LaL into the viral genome. The resultant virus would be expected to contain the floxed a sequence as its unique packaging signal.

Construction and structure of HSV-1 LaL: recombinant HSV-1 containing a unique a sequence, which is surrounded by loxP sites. In order to create HSV-1 LaL, we used a five-cosmid set covering the entire HSV-1 sequence except the a sequences (4, 8). Since disruption of the UL44 locus (encoding gC) does not impair virus replication, we have introduced a fragment from pA-LaL containing the a sequence surrounded by the loxP sites into the unique XbaI site of this locus, harbored by cos56. This floxed sequence is in fact larger than the strict a sequence, since it is originated from an L-S junction and contains 156 and 615 bp from the flanking b and c fragments, respectively. The modified cosmid, named cos56LaL, was cotransfected with cos6Δa, cos14, cos28, and cos48Δa into BHK-21 cells (Fig. 2A). At 4 days posttransfection a single plaque was isolated, presumably resulting from homologous recombination between the overlapping sequences of the five cosmids. This virus, which was named LaL (loxP-a-loxP), was then amplified, and the structure of its genome was confirmed by Southern blotting (data not shown). Due to the insertion of the cleavage-packaging a sequence in the ectopic UL44 locus, the genomic structure of LaL virus is expected to differ from that of wild-type HSV-1. In the case of HSV-1 LaL, the genomic termini should correspond to the disrupted UL44 sequences, thus cleaving the Ul1 segment into two parts (Ul1 and Ul2), as shown in Fig. 2B. Segment Ul1 contains genes UL1 to UL43, whereas Ul2 contains genes UL45 to UL56. Since the orientation of the a sequence in cos56LaL is Ul1-[-a(c)-a(b)]-loxP-Ul2, the L2 end of the cleaved LaL genome is expected to parallel a bona fide L end [a(c)-a(b)]-loxP-Ul2, whereas the LaL L1 end [Ul1)[-a(c)-a)] corresponds to an S terminus. BamHI digestion of the LaL viral genome should release new termini of about 1 kbp (L2 end) and 0.7 kbp (L1 end). The analysis of viral genomic ends was performed by Southern blotting of DNA extracted from the cytoplasm of infected cells, since the viral DNA present in this fraction corresponded to the cleaved and packaged viral DNA. Southern blots of 17+ and LaL genomes, after BamHI digestion and hybridization with a loxP-a-loxP probe (Fig. 2C), showed that LaL virus contained two novel genomic ends at the expected sizes. In addition, the novel L2 terminus also exhibited the length heterogeneity characteristic of normal L termini, furnishing a family of bands resulting from amplification
FIG. 2. Construction and structure of HSV-1 LaL. (A) Principle of HSV-1 LaL construction using the cosmid set covering the entire HSV-1 sequence with the a sequence deleted (4, 8) and the modified cos56LaL. (b) and (c), short sequences of b and c segments of, respectively, 156 and 615 bp introduced with the a sequence between the loxP sites. (B) Genomic structure of HSV-1 17+ and HSV-1 LaL. In the latter virus, cleavage occurs at the UL44 locus, breaking UL into two segments, UL1 and UL2. Amplification of the a sequence at the genomic ends is also shown. B, BamHI sites used in this study. (C) Southern blot analysis of HSV-1 17+ and HSV-1 LaL genomic ends, after BamHI digestion and hybridization with a loxP-a-loxP probe. Molecular weights are indicated. Empty circles indicate fragments not reproducibly observed with another a probe (data not shown), probably representing unspecific labeling.
of the \(a\) sequence (5, 19, 20). The L1 terminus presented a single fragment, paralleling the behavior of bona fide S termini. Additional fragments were not reproducibly observed (data not shown) and most probably represent unspecific hybridization. Moreover, failure to observe (in HSV-1 L1 DNA) fragments of 3.4 and 2.9 kbp, corresponding respectively to the \(BamH\) \(I\) and \(S\) ends of wild-type HSV-1, strongly suggests that no regeneration of \(a\) sequence occurred at the \(L-S\) junctions, between the \(b\) and \(c\) sequences. Therefore, due to cleavage at the unique \(a\) sequence introduced into the ectopic UL44 locus, the singular LaL virus presents a novel genome structure, with no internal repeats of \(a\) signals, and carrying spatially permuted blocks of genes.

**Replication of HSV-1 LaL.** In order to study the replication properties of HSV-1 LaL, we conducted growth curve experiments with this virus and control HSV-1 17+ on TE-671 and on TE-CRE30 cells. TE-671 or TE-CRE30 cells were infected with HSV-1 LaL or HSV-1-17+ at an MOI of 0.1 PFU/cell. At indicated times infections were stopped and virus yields (cell-associated plus released particles) were estimated by titration on Vero cells and plotted.

![Graph showing growth kinetics of HSV-1 LaL and HSV-1 17+ viruses.](image)

FIG. 3. Growth kinetics of HSV-1 LaL and HSV-1 17+ viruses. TE-671 or TE-CRE30 cells were infected with HSV-1 LaL or HSV-1-17+ at an MOI of 0.1 PFU/cell. At indicated times infections were stopped and virus yields (cell-associated plus released virus) were estimated by titration on Vero cells and plotted.

step the replication of HSV-1 LaL was inhibited on Cre-expressing cells, we analyzed the status of viral DNA at different times postinfection using PFGE and hybridization with an HSV-1 DNA probe (Fig. 4). In TE-671 cells, both the replication intermediates (which remained in the wells) and the cleaved genomic units (which migrated at around 150 kbp) could be observed from 24 to 72 h postinfection. On the other hand, in TE-CRE30 cells, although newly replicated viral intermediates still accumulated in the wells during infection, no release of unit length monomers was detectable. These results indicated that LaL DNA synthesis took place but that replication intermediates were not cleaved in Cre-expressing cells, strongly suggesting that growth inhibition resulted from absence of cleavage.

To further confirm the latter result, we studied cleavage of HSV-1 LaL concatemers by analyzing the free viral termini generated both in TE-671 cells and in TE-CRE30 cells. To this, total DNAs extracted from both types of infected cells were digested with \(BamH\) and hybridized with a probe corresponding to the fragment \(loxp\)-\(a\)-\(loxP\) (Fig. 5A). This allowed detection of viral genomic termini, as shown in Fig. 2C. In TE-671 cells, two fragments of about 1 and 0.7 kbp, respectively, corresponding to free genomic termini, were readily detected. The 1-kbp end appeared much more abundant, and the most likely explanation of this is that only \(L\) ends (in our case, \(L2\) ends) are present on concatemeric structures (31), in agreement with an oriented direction for the cleavage process (6, 17). Conversely, on TE-CRE30 cells, we failed to observe these free viral genomic termini, confirming that cleavage of HSV-1 LaL concatemers was specifically inhibited in such cells. This experiment revealed, however, the presence of a ladder of \(loxP\)-\(a\)-\(loxP\) containing fragments in TE-CRE30 cells, which required further investigation.

**Status of a sequence during HSV-1 LaL replication.** In TE-671 cells, the most likely explanation for the ladder revealed by the \(loxP\)-\(a\)-\(loxP\) probe (Fig. 5A) was the presence of multiple copies of the \(a\) sequence, both at \(L2\) ends of the cleaved genomes (as shown in Fig. 2) and at the \(L2\) end and the \(L1-L2\) junctions on the concatemers. In Cre-expressing cells, however, it was unlikely that the family of bands corresponded to free ends containing multiples copies of \(a\), since free viral \(L1\) or \(L2\) ends were actually not detected at all. In these cells, specific Cre-mediated recombination at the \(loxP\) sites on the viral genomes would be expected to result in the excision of circles that the residual infectious virus released by HSV-1 LaL-infected TE-CRE30 cells was passaged again on the same cells but we were unable to detect infectious particles, suggesting that the background virus had not regenerated nonfloxed \(a\) signals (data not shown).

**Newly replicated LaL DNA is not cleaved into unit length monomers on TE-CRE30 cells.** In order to determine at which

![Image](image)

FIG. 4. Cleavage of HSV-1 LaL replication concatemers. Southern blot analysis of total DNA extracted from LaL-infected TE-671 or TE-CRE30 cells at the indicated times, after PFGE and hybridization with HSV-1 probe. Replication intermediates accumulated in the wells, whereas the cleaved unit length genomes migrated around 150 kbp.
containing the a sequence, as previously described with ampli-
con A-LaL. Site-specific recombination events could have oc-
curred either at an immediate-early time in infection, after end
joining of the input genome (25), or once the viral concatemers
were generated during replication. Both situations would bring
loxP sites closer, compared with their relative distance on the
linear genome. If site-specific deletion had taken place on
previously amplified
a sequences whenever amplification had
occurred, the deleted
a sequences should have been present in
circular structures harboring multiple copies of the
a sequence. It was thus possible that at least some of the fragments gen-
erated on TE-CRE30 cells (Fig. 5A) resulted from such dele-
tions. To test this hypothesis, we analyzed LMW DNA ex-
tracted from both types of infected cells by Southern blot
analysis, using a loxP-a-loxP probe, as previously described.
The ladder of DNA molecules containing the a signal, previ-
ously observed with total DNA, was again detected (Fig. 5B)
but only in LMW DNA isolated from infected TE-CRE30
cells, demonstrating that these molecules were excised from
virus DNA by site-specific recombination. Although we can-
not exclude the possibility that some recombination could
occur between loxP sites on TE-671 cells, we were unable to
detect it. Further observations confirmed that the molecules
extracted in LMW DNA from LaL-infected TE-CRE30 cells
were circular. First, the electrophoretic mobility of these frag-
ments was modified following BamHI digestion of LMW DNA
extracts. As indicated in Fig. 2B, only one BamHI site is
present between the two loxP sites surrounding the a signal,
allowing linearization of the circular products of site-specific
recombination. Second, it is of interest to note that if the a
sequences present in the excised circles were subsequently
cleaved by the viral machinery, BamHI digestion of the linear-
ized fragments should produce a small fragment of roughly 0.65
kbp, corresponding to the size of the sequence between the
potential a cleavage site and the BamHI site. In fact, we never
detected such a fragment during these experiments (Fig. 5 and
data not shown), indicating that a sequences harbored by these
molecules remained uncleaved.

Fraction of floxed a sequences can remain unexcised in
replication concatemers. The analysis of LMW DNA showed
that at least part of the a-carrying fragments observed in Fig.
5A represented circles that had been excised from viral DNA
by site-specific deletion. It remained possible, however, that
some of them represented amplified a sequences at the L1-L2
junctions of concatemers, having escaped Cre-mediated re-
combination. To test this possibility, PFGE analysis was per-
fomed with TE-671 and TE-CRE30 cells infected with HSV-1
LaL. After total DNA blotting, membranes were hybridized
first with an a probe (A); then the hybridized a probe was removed, and the filter was hybridized a second time using a BamHI digested HSV-1 DNA probe (B).

FIG. 5. Status and fate of the a sequence of HSV-1 LaL in TE-671 and
TE-CRE30 cells. (A) Southern blot analysis of total DNA extracted from TE-671
or TE-CRE30 cells that had been infected 30 h before by HSV-1 LaL at 0.1
PFU/cell. DNAs were BamHI digested, transferred, and hybridized using a
loxP-a-loxP probe. (B) Southern blot analysis of LMW DNA. LMW DNA was
extracted from TE-671 or TE-CRE30 cells that had been infected with HSV-1
LaL 30 h earlier, at 0.1 PFU/cell. Extracts were digested with BamHI (BamHI)
or not digested (ND) before electrophoresis, transfer, and hybridization with a
loxP-a-loxP probe. Molecular weights are indicated.

FIG. 6. PFGE of HSV-1 LaL-infected TE-671 or TE-CRE30 cells was performed at indicated times postinfection. After transfer, the filters were hybridized a first
time using an a probe (A); then the hybridized a probe was removed, and the filter was hybridized a second time using a BamHI digested HSV-1 DNA probe (B).
than half of the a sequences were deleted (data not shown). This result provided independent confirmation that most of the floxed a sequences were actually deleted in Cre-expressing cells but also showed that some of them could escape from recombination and remained in the replication concatamers. Taken together, these results indicate that the family of BamHI bands observed on TE-CRE30 cells (Fig. 5) originated from both the circular products of site-specific recombination and, in smaller proportion, nondeleted L1-L2 concatameric junctions.

Accumulation of B-type capsids in HSV-1 LaL-infected TE-CRE30 cells. In order to investigate whether Cre-expressing cells allowed normal assembly of HSV-1 LaL capsids, TE-671 and TE-CRE30 cells were infected at a low MOI (0.5 PFU/cell) with HSV-1 LaL, fixed the following day, and analyzed by electron microscopy (Fig. 7A). In TE-671 cells, A-, B-, and C-type capsids were readily observed in the nucleus (Fig. 7A, a, b, and c) while enveloped capsids were observed in the cytoplasm and outside of the cells (Fig. 7A, d). In TE-CRE30 cells (Fig. 7A, e to h), capsids were very abundant in the nucleus. Most of the capsids on TE-CRE30 cells resemble B-type capsids, and many appear to form clusters (Fig. 7A, f, g, and h). Several empty A capsids were also observed, but DNA-containing C capsids were exceptional. These C capsids most likely correspond to the packaged genomes of background virus that escaped Cre recombination, previously observed during growth time course experiments. No major morphological difference between the B-type capsids generated in both types of cells was noted, and, in particular, B capsids presented the same angularity in Cre-expressing cells as in control cells, which corresponded to small-core B capsids (9).

To further analyze the relative proportions of the different type of capsids generated under both conditions, lysates of TE-671 or TE-CRE30 cells infected at a MOI of 0.1 were subjected to 15 to 50% sucrose gradient centrifugation, and capsids were viewed as visible light-scattering bands (Fig. 7B). In LaL-infected TE-671 cells, the three capsid forms were readily observed, with B being the most prominent, while C and A capsids were seen in approximately equal ratios. In LaL-infected TE-CRE30 cell lysates, we observed large numbers of B capsids as well as a smaller though significant amount of C capsids. On the other hand, no C capsids were detected using this approach. Thus, two independent approaches revealed that B-type capsids were normally assembled in Cre-expressing cells in normal amounts but that LaL viral DNA was not packaged into them.

**HSV-1 LaL capsids are functional in Cre-expressing cells.** The last step of this study was to determine if all the proteins involved in cleavage-packaging and with capsid assembly that were produced by HSV-1 LaL virus on TE-CRE30 cells were functional. To this end, we studied whether this system allowed normal packaging of amplicon DNA and for capsid assembly. Using this approach, two independent approaches revealed that normal packaging of amplicon DNA did occur in HSV-1 LaL-infected TE-CRE30 cells. This experiment thus confirmed that all the trans-acting viral functions required for cleavage-packaging of virus DNA and for capsid assembly were functional in HSV-1 LaL-infected TE-CRE30 cells and strongly suggest that the reason the HSV-1 LaL is not packaged in these cells is due to the site-specific deletion of the floxed cis-acting a signals.

**DISCUSSION**

To our knowledge, this is the first study reporting the impact of in situ deletion of the essential cis-acting packaging signals from HSV-1 during its replication. In this work, we have exploited the potential of a site-specific recombination system to delete the unique a sequence carried by a recombinant virus, which is flanked by two parallel loxP sites, in cells expressing Cre recombinase activity.

HSV-1 LaL is able to grow to titers exceeding 10⁶ PFU/ml in control cells, in spite of the presence of the two loxP sites surrounding the ectopic single packaging signal. The genome structure of LaL virus is essentially identical to that of the HSV-1::L-SΔaa construct of Martin and Weber (15), except that the single a sequence of LaL was inserted into the gC locus gene instead of the TK gene. As described for HSV-1::L-SΔaa, we also observed that LaL virus can invert, giving rise to concatamers containing L components in both orientations as wild-type HSV-1. However the two arrangements with L components (U1+1 plus U1+2) in inverted orientations, should be excluded during encapsidation, since they possess a sequences that are both spaced improperly (221 and 83 kbp) and juxtaposed incorrectly for normal packaging of viral DNA (data not shown). Thus, fully half of the replicated DNA generated by LaL virus (as for HSV-1::L-SΔaa) during infection appears wasted, which may contribute to the impairment by 1 log unit of HSV-1 replication compared with HSV-1 17+ at 4 to 5 days postinfection (Fig. 3).

HSV-1 LaL growth is, on the other hand, severely inhibited in Cre-expressing TE-CRE30 cells, and all of the observations reported here indicate that this inhibition results only from Cre-mediated site-specific deletion of a sequences. Thus, PFGE showed that newly synthesized replication intermediates were not cleaved into genomic units, while electron microscopy and sucrose gradient analysis revealed that most capsids accumulating in these cells were B-type capsids. Moreover, all transacting functions encoded by LaL genes, including the replication machinery, the structural proteins that assemble into capsids, and the cleavage-packaging proteins, were functional in the TE-CRE30 cell context, as shown by the amplification and packaging of plasmids harboring one origin of virus replication and one nonfloxed a sequence.

The fate and status of the floxed packaging signal a in both types of cells was further studied. The fact that this single sequence was amplified in the control cell line is not surprising and has already been described (15). Several lines of evidence indicate that L2 ends can act as bona fide wild-type L ends. Thus, (i) they contain multiple repetitions of the a sequence and (ii) L2 ends can be readily observed in concatemer virus DNA undergoing oriented cleavage. On the other hand, L1 ends of LaL do not contain amplified a signals and they are not readily found as free ends on the viral concatamers, indicating that they behave as bona fide S ends.

It is very interesting to note that high-level amplification of a sequences also took place in TE-CRE30 cells. To explain directional packaging of HSV-1 DNA, Deiss et al. advanced two alternative models. According to the double-strand-break-and-gap-repair model, amplification of the a sequence occurs during or after the cleavage process, while according to the directional-cleavage model, cleaved a sequences are already amplified (6). Our results clearly support the second hypothesis, as LaL a sequences appeared to be highly amplified in TE-CRE30 cells although no cleavage of concatamers could be
detected, suggesting that extensive amplification of this sequence occurred early before or during viral replication, independently of the cleavage process. It is possible that amplification of the a sequence occurs during end joining of the viral genome (35) or at the same time as the inversion of L components, which also takes place early during infection (2). Since double-strand breaks introduced by heterologous nuclease are known to induce recombination events in HSV-1 DNA.
(30), it is conceivable that breaks created by the Cre recombinase in the LaL virus may serve as initiating sites for the amplification of the a sequences present in the excised circles. However, for different reasons we do not believe that the observed a amplification results from Cre recombination. First, Cre recombination occurs through the formation of a synaptic complex, implying the involvement of the two loxP sites and four recombinase molecules (see reference 12 and references within), and it seems unlikely that in that type of structure, factors allowing a amplification could enter. Moreover, Cre recombination implies single-strand breaks. Second, the levels of a amplification in the presence and in the absence of Cre recombinase were similar (Fig. 5A), which supports the idea that this amplification is not a consequence of Cre-loxP recombination.

In TE-CRE30 cells, deletion of the floxed a sequence of HSV-1 LaL was enough to strongly inhibit the cleavage-packaging process, by more than 3 orders of magnitude. However, by PFGE and Southern blotting, we were able to detect a proportion of a signals that have escaped from site-specific recombination and remained in the concatemers. It is possible that these correspond to isolated a signals, separated by 300 or more kbp, that were unable to target efficient cleavage packaging of the concatemers. The fact that most of the a sequences were deleted is remarkable in the context of a virus such as HSV-1, whose DNA replication starts very soon after infection.

Deletion of a sequences resulted in accumulation of uncleaved replication concatemers and of B-type capsids. This phenotype is commonly found with most viruses mutated in genes implicated in cleavage packaging, such as UL6, UL15, UL17, UL28, UL32, and UL33 (reviewed in references 13, 26, 29, and 36). The full set of cleavage-packaging proteins is, however, present and functional in HSV-1 LaL-infected TE-CRE30 cells, indicating that their presence is not enough to target expulsion of the scaffold proteins from B capsids. This could mean that this event requires, in addition to the cleavage-packaging proteins, the interaction between B-type capsids and the concatemers via the a sequence, in a very concerted mechanism. A few studies have advanced the possibility that packaging occurs via an early-intermediate designated procapsid, during capsid maturation (22, 23, 28). According to this point of view, B-type capsids would correspond to dead-end products, having engaged in but failed in the packaging process. Although we have not directly addressed this question, the fact that B-type capsids also accumulate when only the cis-acting element is lacking suggests that B capsids are the direct precursors for packaging and that interaction with the viral replication intermediates via the packaging signals is required for the assembled B capsids to engage in the process of degradation of the core protein. However, we cannot rule out the possibility that the deleted a sequences present on the small circles or the few a sequences present on the replication concatemers can still be recognized by the cleavage-packaging machinery and be brought to the procapsids, thereby triggering protease activity, also resulting in the accumulation of the B-type capsids. In other respects, the fact that smaller than normal amounts of A capsids are observed in Cre-expressing cells is consistent with the notion that A capsids result from aborted packaging reactions. Again, the low density of a signals in replication concatemers in TE-CRE30 cells could result in less-frequent interactions between capsids and viral DNA, thus reducing the number of capsids that engage in packaging.

The fact that the a sequences on the circular molecules excised from the concatemers through site-specific recombination appeared uncleaved contrasted with previous observations of specific a cleavage on plasmids harboring a-a junctions after superinfection with HSV-1 (21). However, the authors also indicated that in similar experiments using other plasmid also carrying two complete copies of the a sequence, only a minor portion of the plasmids were cleaved. These experiments are, however, not directly comparable with ours, since the amount of plasmids introduced by transfection is much larger than the amount of circles excised by site-specific recombination. Although we cannot rule out the possibility that the binding of Cre recombinase on the loxP sites could impair recognition of the DNA, our working hypothesis is that cleavage-packaging of virus DNA requires the concerted interaction of B-capsids with a signals located in the replication concatemers, and that isolated circles containing these signals cannot interact in a functional manner with the cleavage-packaging machinery or with capsids.

Extensive comparative studies of the maturation of HSV-1 LaL capsids, the status of the scaffold protein, and the interaction of capsids with the cleavage-packaging machinery, both in TE-671 and in TE-CRE30 cells, could provide a new approach to the understanding of these complex steps of HSV-1 replication. Lastly, results presented in this study indicate that HSV-1 LaL could be used as helper virus in TE-CRE30 cells, as a new alternative system for producing large amounts of ampiclon vectors only slightly contaminated by helper virus. Elaboration of such a system is in progress.

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