Knockdown of DNA Ligase IV/XRCC4 by RNA Interference Inhibits Herpes Simplex Virus Type I DNA Replication*

Isabella Muylaert and Per Elias

From the Institute of Biomedicine, Department of Medical Biochemistry and Cell Biology, Göteborg University, SE-405 30 Göteborg, Sweden

Herpes simplex virus has a linear double-stranded DNA genome with directly repeated terminal sequences needed for cleavage and packaging of replicated DNA. In infected cells, linear genomes rapidly become endless. It is currently a matter of discussion whether the endless genomes are circles supporting rolling circle replication or arise by recombination of linear genomes forming concatemers. Here, we have examined the role of mammalian DNA ligases in the herpes simplex virus, type I (HSV-1) life cycle by employing RNA interference (RNAi) in human 1BR.3.N fibroblasts. We find that RNAi-mediated knockdown of DNA ligase IV and its co-factor XRCC4 causes a hundred-fold reduction of virus yield, a small plaque phenotype, and reduced DNA synthesis. The effect is specific because RNAi against DNA ligase I or DNA ligase III fail to reduce HSV-1 replication. Furthermore, RNAi against DNA ligase IV and XRCC4 does not affect replication of adenovirus. In addition, high multiplicity infections of HSV-1 in human DNA ligase IV-deficient cells reveal a pronounced delay of production of infectious virus. Finally, we demonstrate that formation of endless genomes is inhibited by RNAi-mediated depletion of DNA ligase IV and XRCC4. Our results suggest that DNA ligase IV/XRCC4 serves an important role in the replication cycle of herpes viruses and is likely to be required for the formation of the endless genomes early during productive infection.

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1 and Tables S1 and S2.

2 To whom correspondence should be addressed: Institute of Biomedicine, Dept. of Medical Biochemistry and Cell Biology, Göteborg University, Box 440, SE-405 30 Göteborg, Sweden. Tel.: 46-31-7863486; Fax: 46-31-416108; E-mail: per.elias@medkem.gu.se.

3 The abbreviations used are: HSV-1, herpes simplex virus, type I; RNAi, RNA interference; FBS, fetal bovine serum; siRNA, small interfering RNA; MOI, multiplicity of infection; PFU, plaque-forming units; p.i., post-infection; PBS, phosphate-buffered saline.
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however, exist some functional overlap between the three mammalian DNA ligases. This is perhaps best illustrated by the observation that proliferating fibroblasts can be derived from knock-out mice for DNA ligase I, although they display an accumulation of DNA replication intermediates and increased genomic instability (20).

We have used RNAi directed against DNA ligases I, III, and IV in human fibroblasts to examine the effects on HSV-1 DNA replication. Interestingly, only RNAi against DNA ligase IV and its partner XRCC4 caused a significant reduction of HSV-1 DNA synthesis and virus production. In contrast, adenovirus replication was not affected. We suggest that DNA ligase IV is needed early to promote formation of circular templates for DNA replication.

EXPERIMENTAL PROCEDURES

Cells—1BR.3.N (ECACC), 293 (ATCC/LGC) and GM16089 (Coriell Cell Repositories), which has a R278H mutation in the DNA ligase IV gene (21), were propagated in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and nonessential amino acids (Invitrogen). The untransformed fibroblast cell line GM17523 (Coriell Cell Repositories), which is a compound heterozygote resulting from E566K and R771W mutations in the DNA ligase IV alleles (22), and GM16097 cell line (Coriell Cell Repositories), a compound heterozygote from E566K and R771W mutations in the DNA ligase I gene (14, 23), were grown in Dulbecco’s modified Eagle’s medium containing 15% FBS and nonessential amino acids. BHK-21 cells (ATCC) were cultured in Glasgow minimum essential medium (Invitrogen) supplemented with 10% FBS.

siRNA Transfection—RNA oligonucleotides were directed against the following target sequences: DNA ligase I, 5’-AAGGGCAAGACAGCAGGGCC; DNA ligase III, 5’-AAC-UGCAACCCAGAUGAUAUG; DNA ligase IV, 5’-AAGCACA-GACAAAAAGGGGAA; DNA ligase IVmut, 5’-AAAGCGAA-AACACAGAGGGGCAA; XRCC4, 5’-AAUCUUUGCAGAC-ACCUAA; and XRCC4b, AUAUGUUGUGAACUGAGAd-TdT. The sequence XRCC4b was from Ahnesorg et al. (24).

Double-stranded siRNA molecules were purchased from Dharmacon and contained a dT-dinucleotide at the 3’ ends. Cell monolayers in 12-well plates were transfected with 450 pmol of siRNA duplexes by using oligofectamine (Invitrogen) as recommended by the manufacturer. Mock transfected cells were treated with oligofectamine only.

Quantitative Real Time PCR—Reverse transcription was performed using the BD Bioscience/Clontech Advantage RTfor-PCR kit as described by the manufacturer. Quantitative PCR was performed on duplicate samples using Corbett Research Rotorgene 3000. Fluorescence measurements were made after the elongation step in each cycle. Each cDNA sample was quantified in duplicate and normalized to β-actin cDNA. Primers were designed to produce a PCR product spanning the RNAi binding sites in DNA ligase mRNAs, and β-actin mRNA was used as a reference. The analyses were performed by TATAA Biocenter, Lundberg Laboratory, Göteborg, Sweden.

Protein Analysis—At 72 h post-transfection, 1BR.3.N monolayers were harvested with SDS lysis solution containing 75 mM Tris-HCl, pH 6.8, 0.6% SDS, 12% glycerol, 1 mM EDTA, 1% β-mercaptoethanol, 10 mM dithiothreitol, and Complete protease inhibitors mix (Roche Applied Science). The protein lysates were subjected to electrophoresis on 4–15% SDS-PAGE gradient gels and transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were probed with primary mouse monoclonal or polyclonal antibodies to human DNA ligase I (ab6165; Abcam), human DNA ligase III (ab587; Abcam), human XRCC4 (ab2857; Abcam), and a synthetically produced β-cytoplasmic N-terminal peptide (ab6276; Abcam). A secondary goat anti-mouse IgG conjugated to horseradish peroxidase (Pierce) was used for detection. DNA ligase IV is present in very small amounts in the cell, and as recommended by the supplier of the antibody, immunoprecipitation was therefore performed prior to the Western blot analysis. The cells were treated with Triton lysis solution (0.5% Triton X-100, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and Complete protease inhibitors; Roche Applied Science) and immunoprecipitated using rabbit polyclonal antibodies to human DNA ligase IV (SP1275, Acris). The immunoprecipitates were collected using insoluble Protein A from Staphylococcus aureus Cowan strain I (Sigma). Western blot analysis was performed as described above using primary rabbit polyclonal antibodies to human DNA ligase IV (ab6145; Abcam) and secondary goat anti-rabbit IgG conjugated to horseradish peroxidase (Pierce). For detection of UL30, siRNA-treated cells were infected with HSV-1 at a multiplicity of infection (MOI) of 8 plaque-forming units (PFU)/cell, in the presence or absence of phosphonoacetic acid at 400 μg/ml. At 12 h p.i., the cells were collected with SDS lysis solution and subjected to Western blot analysis, as described above, using rabbit polyclonal antibodies to HSV-1 UL30. All of the protein bands were detected by chemiluminescense.

Virus Assays—HSV-1 (Glasgow strain 17 syn+) titers were determined by plaque assay on BHK cells. Infected monolayers were overlaid with minimum essential medium containing 2% FBS and 0.5% agarose (Invitrogen). After fixation of the cells with 7% formaldehyde (Sigma) and removal of the agarose overlay at 5 days post-infection, the plaques were visualized by staining with 1.3% crystal violet (Sigma) in 20% ethanol. Adenovirus (wt900) was provided by Göran Akusjärvi (Uppsala University). Titers were determined by plaque assay on 293 monolayers grown on collagen-treated plates. Infected cells were overlaid with minimum essential medium containing 2% FBS and 0.4% agarose. The plaques were visualized at 7 days post-infection as described above.

To measure virus production, the cells were treated with siRNA for 48 or 72 h. The cell monolayers were then infected with either HSV-1 or adenovirus at a MOI of 8 PFU/cell. The cell culture supernatants were collected at the indicated times, clarified, and stored at −80 °C. The virus titers were determined by plaque assay.

Growth curves of HSV-1 on human cells were performed as follows. Monolayers of 1BR.3.N, GM17523, GM16089, and GM16097 cells were infected with HSV-1 virus at a MOI of 8 PFU/cell. After 1 h, the inoculum was removed, and the mono-
layers were washed with Dulbecco’s modified Eagle’s medium followed by incubation with 1 ml of Dulbecco’s modified Eagle’s medium containing 2% FBS. The cell culture supernatants were harvested at 0, 4, 8, 12, 16, and 24 h.p.i., clarified by centrifugation, and stored at −80 °C. Titers were determined by plaque assays in BHK cells.

Growth curve analyses on siRNA-treated cells were performed at 72 h post-transfection, as described above, and cell culture supernatants were harvested at 0, 4, 6, 8, 10, 12, and 24 h.p.i.

Plaque Immunostaining—At 72 h post-transfection, 1BR.3.N cells were infected with HSV-1 for 1 h and overlaid with minimum essential medium containing 2% FBS and 0.5% agarose. After 3 days of incubation, the agarose overlayers were carefully removed, and the cells were fixed with cold isopropanol for 10 min at 4 °C. Monolayers were washed twice with phosphate-buffered saline (PBS), incubated with rabbit polyclonal antibodies to HSV-1 ICP8 (diluted 1:5,000 in PBS) for 30 min at 37 °C, washed three times with PBS, incubated with biotinylated goat anti-rabbit IgG (diluted 1:200 in PBS; Pierce), and washed as before. Monolayers were then incubated with the ImmunoPure ABC alkaline phosphatase staining kit (Pierce) for 30 min at room temperature, and plaques were visualized by using the ready-to-use 1-step™ nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (Pierce). To terminate the reaction, the cells were rinsed with destilled water.

DNA Replication Assays—Monolayers of 1BR.3.N were treated with RNAi for 72 h. The cells were then infected with HSV-1 at a MOI of 8 PFU/cell. DNA was isolated 20 h.p.i. using 500 µl/well of 1.2% sodium dodecyl sulfate, 50 mM Tris-HCl, pH 7.5, 4 mM EDTA, 4 mM CaCl2, 0.2 mg/ml proteinase K, and 10 µg/ml CT- DNA for 2–3 h at 37 °C. Total DNA was prepared by phenol extraction and ethanol precipitation. The DNA samples were cleaved overnight with BamHI and subjected to electrophoresis on 0.9% agarose gels, followed by alkaline transfer to Hybond-H+ membranes, as recommended by the supplier (Amersham Biosciences). The membrane was hybridized with a 1.0 kb radiolabeled probe (Megaprime labeling system; Amersham Biosciences) derived from a BamHI fragment from a 1.0-kb radiolabeled probe (Megaprime labeling system; Amersham Biosciences). The membrane was hybridized with a 1.0 kb radiolabeled probe (Megaprime labeling system; Amersham Biosciences) derived from a BamHI fragment from Melanoma cell line 1BR.3.N. The knockdown effects were monitored by real time PCR and Western blotting. Quantitative PCR using β-actin mRNA as a control showed a significant and specific reduction of mRNA levels for DNA ligases I, III, and IV at 48 h p.i. post-transfection (Table 1). The levels of β-actin mRNA were not affected (results not shown). A very significant and specific reduction in the amount of DNA ligase I, III, and IV after treatment with siRNA for 72 h could also be demonstrated by Western blot analysis (Fig. 1). In a similar way, we investigated the effects of RNAi against the DNA ligase IV co-factor XRCC4 (Fig. 1 and Table 1.). The concentrations of siRNA and lipids used in the transfection experiments throughout these investigations were chosen to avoid giving rise to altered morphology of the cells and limit nonspecific toxic effects.

RNAi against DNA Ligase IV and XRCC4 Inhibits HSV-1 Replication—We first examined effects of RNAi against the DNA ligases on the production of infectious virus in 1BR.3.N cells. We found that RNAi against DNA ligase I and DNA ligase III had no effect on virus production, whereas treatment of cells with RNAi against DNA ligase IV for 48 h caused an 8-fold reduction on virus yield. If treatment with siRNA was extended to 72 h before infection with HSV-1, the reduction of virus production was 19-fold after 48 h of siRNA treatment and 100-fold after 72 h of siRNA treatment (Table 1). These observations also indicated that maximal reduction of protein levels were achieved after 3 days of treatment with siRNA. Serial dilutions of mock transfected cell extracts were made to determine the levels of DNA ligase IV and XRCC4 in cells treated with siRNA for 72 h. We estimate a

### TABLE 1

| DNA Ligases and Herpes Virus Replication |
|----------------------------------------|
| **RNAi** | **mRNA**<sup>a</sup> | **Virus yield** |
|          | (exp. 1)<sup>b</sup> | **PFU/ml** |
| No siRNA | 100 | 5.8 ± 1.8 × 10<sup>6</sup> | 2.4 ± 0.1 × 10<sup>6</sup> |
| Ligase I | 30  | 3.5 ± 1.0 × 10<sup>6</sup> | 1.9 ± 0.1 × 10<sup>6</sup> |
| Ligase III | 18  | 3.5 ± 1.0 × 10<sup>6</sup> | 2.0 ± 0.1 × 10<sup>6</sup> |
| Ligase IV | 45  | 8.0 ± 2.0 × 10<sup>6</sup> | 2.2 ± 0.3 × 10<sup>6</sup> |
| XRCC4 | 22  | 4.5 ± 0.5 × 10<sup>6</sup> | 1.7 ± 0.3 × 10<sup>6</sup> |
| Ligase IV/XRCC4 | ND<sup>c</sup> | ND<sup>d</sup> | ND<sup>d</sup> |

<sup>a</sup> mRNA was quantified by quantitative real time PCR at 48 h post-RNAi transfection. The values indicate the percentages relative to the amount of mRNA present in cells not treated with siRNA.

<sup>b</sup> 1BR.3.N monolayers were transfected with siRNA for 48 h followed by HSV-1 infection at a MOI of 8 PFU/cell for 20 h. Titers were determined by plaque assay on BHK cells.

<sup>c</sup> 1BR.3.N monolayers were transfected with siRNA for 72 h, followed by HSV-1 infection at a MOI of 8 PFU/cell for 20 h. Titers were determined by plaque assay on BHK cells.

<sup>d</sup> ND, not determined.

### RESULTS

RNAi-mediated Knockdown of DNA Ligase I, III, and IV—To investigate the role of DNA ligases in the life cycle of HSV-1, we have attempted to specifically knock down the level of these enzymes in human cells using RNA interference. Conserved target sequences in DNA ligases I, III, and IV in mice and men were selected considering suggestions by Elbashir et al. (25). The resulting double-stranded 21-mer oligonucleotides were used to transfect a SV40 T-antigen transformed human fibroblast cell line 1BR.3.N. The knockdown effects were monitored by real time PCR and Western blotting. Quantitative PCR using β-actin mRNA as a control showed a significant and specific reduction of mRNA levels for DNA ligases I, III, and IV at 48 h p.i. post-transfection (Table 1). The levels of β-actin mRNA were not affected (results not shown). A very significant and specific reduction in the amount of DNA ligase I, III, and IV after treatment with siRNA for 72 h could also be demonstrated by Western blot analysis (Fig. 1). In a similar way, we investigated the effects of RNAi against the DNA ligase IV co-factor XRCC4 (Fig. 1 and Table 1.). The concentrations of siRNA and lipids used in the transfection experiments throughout these investigations were chosen to avoid giving rise to altered morphology of the cells and limit nonspecific toxic effects.
70% knockdown of these proteins upon siRNA treatment (supplemental Fig. S1).

We have also used a mutant version of siRNA against DNA ligase IV, Lig IVmut, with three scattered base pair mutations, which does not significantly reduce the levels of DNA ligase IV mRNA. Lig IVmut siRNA only cause a 3-fold reduction in HSV-1 replication under conditions where Lig IV siRNA results in an 80-fold reduction in virus yield (supplemental Table S1). Mutant siRNAs with five or more base pair changes cause no reduction on virus yield (results not shown). We cannot exclude the possibility that siRNA Lig IVmut mentioned above may give rise to a mild RNAi effect.

To ascertain that the RNAi-mediated inhibition was specific, a second siRNA, XRCC4b, against XRCC4 was used. The sequence of this species was derived from a previous study on XRCC4 (24). We found, using siRNA against DNA ligase I as a control, that siRNA against XRCC4 reduced the yield of infectious virus 100-fold (supplemental Table S2).

To confirm these results, we looked at the formation of HSV-1 plaques on 1BR.3.N cells (Fig. 2). Plaques were identified by immunostaining using a rabbit polyclonal antiserum against ICP8. Infected mock transfected cells displayed plaques with a hollow center surrounded by an intensely staining ring of cells expressing ICP8. Monolayers that were transfected with siRNA against either DNA ligase I or DNA ligase III and subsequently infected with HSV-1 exhibited plaques of normal size and morphology. On the other hand, monolayers treated with siRNA against DNA ligase IV and XRCC4 prior to infection with HSV-1 exhibited miniscule plaques (Fig. 2).

DNA Ligase IV Is Not Required for Adenovirus Replication

To further assess the specificity of the RNAi-mediated effects and the viability of cells upon this treatment, we examined the role of DNA ligase IV and XRCC4 for replication of adenovirus. Adenovirus does not form endless concatemers during replication, most likely as a result of inactivation of the NBS-Mre11-Rad50 complex involved in nonhomologous end joining (26). In accordance with this observation, we found only a small reduction of adenovirus replication after treatment of 1BR.3.N cells with siRNA against DNA ligase IV or XRCC4. In contrast, HSV-1 DNA replication was reduced 100-fold (Table 2). This result argues in favor of a specific role for DNA ligase IV and XRCC4 in HSV-1 replication.

Replication of HSV-1 Is Delayed in Cells with Reduced Amounts of DNA Ligase IV or XRCC4—Our results so far indicate a specific role for DNA ligase IV/XRCC4 during replication of HSV-1. We therefore wanted to examine the infectious process in DNA ligase IV-deficient human cells. Such cell lines have been established from patients with Lig IV syndrome: GM17523, an untransformed compound heterozygote cell line, which has a residual activity of DNA ligase IV estimated to be less than 1% (22, 27), and GM16089, a SV40 transformed cell
Initial fate of viral genomes in cells undergoing RNA interference against DNA ligase IV and XRCC4 (Fig. 5). We analyzed the effect of RNAi-mediated knock-down of DNA ligase IV and XRCC4 on replication of adenovirus and herpes simplex virus.

**TABLE 2**

| RNAi                  | Virus yield |
|-----------------------|-------------|
|                       | HSV1*       | Adenovirus* |
| No siRNA              | 1.7 ± 0.2 × 10⁹ | 2.0 ± 0.5 × 10⁷ |
| Ligase IV             | 1.9 ± 0.6 × 10⁹ | 1.4 ± 0.1 × 10⁷ |
| XRCC4                 | 1.4 ± 0.1 × 10⁹ | 9.0 ± 1.0 × 10⁶ |

*1BR.3.N monolayers were transfected with siRNA for 72 h, followed by infection with HSV-1 at an MOI of 8 PFU/cell. Titters were determined by plaque assay on BHK cells.
*1BR.3.N monolayers were transfected with siRNA for 72 h, followed by adenovirus infection at a MOI of 6 PFU/cell. Titers were determined by plaque assay on 293 cells.

A complementary growth curve experiment was performed using RNAi. Here, we found that RNAi directed against DNA ligase IV and XRCC4 caused a substantial delay in the onset of virus production. In contrast, virus replication was normal in cells treated with siRNA against DNA ligase IV (Fig. 4b).

**FIGURE 3.** Inhibition of HSV-1 DNA synthesis by RNAi against DNA ligase IV/XRCC4. 1BR.3.N cells were treated with siRNA against DNA ligase IV and XRCC4 for 72 h, followed by infection with HSV-1 at an MOI of 8 PFU/cell. At 20 h p.i., DNA was isolated and digested with BamHI. The samples were analyzed by Southern blotting using a UL9 probe. The numbers below the autoradiogram indicate the relative amounts of DNA, as determined by phosphorimaging analyses. The sample sizes are indicated by 1x and 2x. The lower panel shows the agarose gels stained with ethidium bromide prior to the Southern blotting procedure. n.d., not determined.

**FIGURE 4.** Growth curves for HSV-1 at high multiplicity of infection. a, monolayers of 1BR.3.N, GM16097 (Lig I mut), GM17532 (Lig IV mut), and GM16089 (Lig IV mut) were infected with HSV-1 at a MOI of 8 PFU/cell. Each graph represents the mean of two independent growth curves for each cell type (“Experimental Procedures”). b, monolayers of 1BR.3.N were transfected with siRNA against DNA ligase I, DNA ligase IV, and XRCC4 for 72 h and subsequently infected with HSV-1 at an MOI of 8 PFU/cell. Growth curves were generated as above.

mutant cells, the rate of virus production appeared to be normal.

GM16097 cells mutated in DNA ligase I revealed no overt phenotype (Fig. 4a). However, we found that HSV-1 exhibited small plaque phenotypes on these cells (results not shown).

DNA Ligases and Herpes Virus Replication

—During the first few hours of an HSV-1 infection, a limited number of linear viral genomes enters the nucleus. It has been noted that the linear genomes may rapidly become endless regardless of whether de novo protein synthesis or DNA replication can be carried out (2–4). We have examined the initial fate of viral genomes in cells undergoing RNA interference against DNA ligase IV and XRCC4 (Fig. 5). We analyzed the structure of virus DNA isolated from cells after restriction enzyme cleavage with BamHI (28). This enzyme cleaves in the terminal repeats as well as the inverted central repeats. It is thus able to generate three fragments, here designated a, b, and c, containing the a sequence. Fragments a and b are 2.9 and 3.4 kb in length, and they are not resolved by agarose gel electrophoresis under these conditions. Fragment c has a length of 6.3 kb. Fragments a and b represent terminal fragments containing the a sequence, and fragment c represents a central fragment containing the internal a sequence repeats. We find that input virus DNA, harvested 1 h after addition to the cells, remains linear as indicated by the presence of all three fragments. In contrast, when virus DNA was harvested 4 h p.i., the amount of fragments a and b was severely reduced, indicating the conversion of the linear genome to an endless, most likely circular, configuration. Interestingly, when virus DNA from cells treated with line (21). For comparison, we also included GM16097, which expresses mutant versions of DNA ligase I (14, 23). Growth curve analyses at high MOIs showed a substantial release of infectious virus from 1BR.3.N cells and GM16097 cells already 8 h after infection (Fig. 4a). At this time, there was no release or very little release of infectious virus from the cells carrying mutant versions of DNA ligase IV. GM17523 displayed a more severe phenotype than GM16089. Thus, at high multiplicity of infection, reduced activity of DNA ligase IV caused a pronounced delay in the release of infectious virus (Fig. 4a). At later times, once virus production had commenced in DNA ligase IV.
siRNA against DNA ligase IV and XRCC4 is examined, we find that the ratio of terminal fragments to internal fragments is very similar to the ratio observed with linear input virus DNA. These results argue that DNA ligase IV and XRCC4 act early during the infectious cycle and that they are needed for conversion of linear DNA to endless form.

**DISCUSSION**

The replication of Herpes simplex virus has several steps that must involve the activity of cellular DNA ligases. The formation of endless genomes may take place within an hour after infection (2, 4). At later times, the joining of lagging strand intermediates and completion of recombination must occur. In addition, the genetically stable virus genome is likely to be a substrate for most cellular DNA repair pathways (29–31). Using RNAi against human DNA ligases I, III, and IV, we provide evidence for a specific role of DNA ligase IV and its co-factor XRCC4 in the HSV-1 life cycle. On the other hand, deletion of neither DNA ligase I nor DNA ligase III appears to affect virus replication. Residual enzyme activity in siRNA-treated cells or the existence of redundant pathways for processing of lagging strand intermediates may account for the lack of an effect of RNAi against DNA ligase I and DNA ligase III.

DNA ligase IV and XRCC4 function only in double-strand break repair by homologous end joining but not in other DNA repair reactions (18, 19). Our results suggest that reduced levels of DNA ligase IV and XRCC4 cause a delay in the onset of virus replication, but once virus replication starts it seems to proceed normally. We also observe that RNAi against DNA ligase IV and XRCC4 prevents the formation of endless genomes early during the infection. It is therefore less likely that DNA ligase IV/XRCC4 would be required for repair of double-stranded breaks during ongoing DNA synthesis, because error-free homologous recombination would be more appropriate in this instance. In fact, homologous recombination is a frequent phenomenon during productive replication of HSV-1 (32). Together, these considerations lead us to propose a model for early HSV-1 replication (Fig. 6). The model, which is a adaptation of the classical model for herpes virus replication, is applicable also to naturally occurring herpes viruses lacking extensive directly repeated sequence at their termini as, for example, is Tupaia herpes virus (33). It is also fully compatible with the results obtained from analysis of the fate of HSV-1 genome in infected cells (2–4). Recent results very strongly indicate that virus genomes may undergo end-to-end ligation to form circular molecules (4). We now suggest that the formation of endless genomes is catalyzed by DNA ligase IV/XRCC4.

DNA synthesis initiated at circular molecules will give rise to products that may become decatenated by topoisomerase II (10). A switch to rolling circle replication is likely to occur later, but the genetic requirements for this event remain unknown. The existence of two distinct phases in HSV-1 replication is also supported by the observation that the initiator protein origin-binding protein/UL9 is only strictly required at early times for HSV-1 DNA replication (34). The model implies that homologous recombination is not required for DNA synthesis. Instead, it can be regarded as a repair function, and the complex structure of HSV-1 replication intermediates may reflect the presence of multiple origins of DNA replication and repeated sequence elements in the HSV-1 genome. It is worth noting that human herpes virus 6, which lacks internally repeated sequences, replicates largely without forming branched intermediates (35).

We have previously found that transfection of cells with linear plasmids containing the HSV-1 origin of DNA replication...
efficiently can provide templates for HSV-1-dependent DNA synthesis (36, 37). This pathway, which proceeds by nonconservative homologous recombination, may be an alternative but less efficient way to support HSV-1 replication, perhaps because of a lack of factors that may target linear DNA to DNA ligase IV/XRCC4 (Fig. 6).

Double-strand break repair by nonhomologous end joining involves a steadily increasing number of proteins. We do not yet know what components, in addition to DNA ligase IV/XRCC4, may contribute to HSV-1 replication. Below, we summarize some observations regarding proteins involved in double-strand break repair and their relationship to HSV-1 replication.

It has recently been found that an undisturbed infection with HSV-1 will activate a DNA damage response as detected by autophosphorylation at ATM S1981 (30, 31). The magnitude of ATM autophosphorylation at ATM S1981 (30, 31). The magnitude of the response is initially low but increases as replication proceeds (30, 31). It appears that the damage response may require active virus replication because a replication defective amplification of DNA ligase IV/XRCC4 on double-stranded breaks is not necessary for circularization the viral genome (45). Finally, it is of interest to note that nonhomologous end joining appears to be a lack of factors that may target linear DNA to DNA ligase IV/XRCC4 (Fig. 6).

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In conclusion, the involvement of DNA ligase IV/XRCC4 in HSV-1 replication when compared with normal mouse embryonic fibroblasts (42). It should perhaps be noted that mouse embryonic fibroblasts appears to support much less efficient replication of HSV-1 when compared with normal human fibroblasts (42). The observation, if valid also in human cells, may indicate that Ku70 may exert an inhibitory effect on replication of the HSV-1 genome, perhaps by competing with as yet unidentified virus and/or host-specific factors. The existence of yet to be identified viral factors has received recent support since it has been demonstrated that the HSV-1 protein ICP4 appears to be necessary for circularization the viral genome (45). Finally, it is of interest to note that nonhomologous end joining appears to play a role also for growth of some bacteriophages (46).

In conclusion, the involvement of DNA ligase IV/XRCC4 in the replication of HSV-1 lends credibility to the classical model for replication of herpes viruses. It also suggests alternative targets for antiviral therapy and may lead to new insights into the organization and regulation of double-strand break repair in mammalian cells.

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