JOURNAL OF VIROLOGY, Sept. 1997, p. 6842–6849 Vol. 71, No. 9
0022-538X/97/$04.00+0

Direct Repeats of the Herpes Simplex Virus *a* Sequence Promote Nonconservative Homologous Recombination That Is Not Dependent on XPF/ERCC4

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Received 4 February 1997/Accepted 3 June 1997

We have examined mechanisms of recombination in mammalian cells infected with herpes simplex virus type 1 (HSV-1). Amplification of plasmids containing a viral origin of replication, oriS, in cells superinfected with HSV-1 revealed that linear DNA could be efficiently converted to templates for replication. Two distinct pathways were observed: imprecise end joining and nonconservative homologous recombination. We noted that direct repeats of the viral *a* sequence promoted efficient nonconservative homologous recombination in BHK cells as well as human repair-proficient 1BR.3N cells and xeroderma pigmentosum group F (XP-F) cells. The reaction gave rise to functional *a* sequences supporting the formation of defective viruses. It did not seem to proceed by single-strand annealing since it occurred in the absence of XPF/ERCC4, the mammalian homolog of the Rad1 endonuclease from *Saccharomyces cerevisiae*. In contrast, direct repeats of a 161-bp nonviral sequence did not take part in nonconservative homologous recombination in XP-F cells. Our results suggest that homologous recombination may be involved in the circularization of viral genomes. Furthermore, they demonstrate that amplification of recombination products supported by HSV-1 allows a direct examination of pathways for double-strand-break repair in human cells.

Replication of the herpes simplex virus type 1 (HSV-1) genome requires seven *trans*-acting viral gene products and at least two types of *cis*-acting sequences: the origins of replication, oriS and oriL; and the directly repeated terminal *a* sequences, which provide the signals for cleavage and packaging (23).

The mechanism for viral replication is not fully understood. It has, however, been noted that the linear genome efficiently circularizes in the nuclei shortly after infection (7, 22). Viral DNA synthesis might then proceed via a theta-type intermediate. Synthesis of HSV-1 DNA is dependent on topoisomerase II during the initial phase of the infectious cycle (11). This observation suggests that decatenation of early replication products might be an essential step in the replication of HSV-1. At later times in the infectious cycle, the formation of long concatemers of directly repeated genomes is seen (29). Rolling circle replication might therefore directly support the formation of a large part of the virus progeny.

The replication of HSV-1 DNA seems to be intimately coupled to homologous recombination. In fact, it has been shown that the frequency of homologous recombination during HSV-1 DNA replication increases more than 10-fold (4, 39). The physiological role of homologous recombination during the viral life cycle is not known. It could be involved in the circularization of viral genomes, and it most likely takes part in the inversion of the U_L and U_S segments of the viral genome (19, 23). Perhaps the primary replication products need extensive recombination-dependent repair in order to give rise to viable progeny. The role of *trans*-acting factors during homologous recombination stimulated by HSV-1 has not been studied.

In this study, we attempted to clarify the relationship between DNA replication and recombination during the viral life cycle. An examination of the topological requirements for efficient replication of oriS-containing plasmids in cells superinfected with HSV-1 revealed that circular DNA replicated better than the corresponding linear molecule. We noted that the linear molecules provided with directly repeated viral *a* sequences at the ends could be efficiently circularized by using two distinct mechanisms: end joining independent of extensive sequence homology and nonconservative homologous recombination. The latter reaction in the yeast *Saccharomyces cerevisiae* has been shown to depend entirely on the *rad1* and *rad10* gene products (13). The mammalian counterparts to these molecules are ERCC4 and ERCC1, respectively (41). These gene products together form an endonuclease that is required also for nucleotide excision repair. Cells from patients with the genetic disease xeroderma pigmentosum of complementation group F (XP-F cells) lack functional XPF/ERCC4 (29a). We have used this observation to examine the role of XPF/ERCC4 during nonconservative homologous recombination in human cells. The results show that direct repeats of one 161-bp sequence of nonviral origin did not take part in nonconservative homologous recombination in XP-F cells. It did so, however, in repair-proficient human cells. In contrast, the HSV-1 *a* sequence promoted efficient circularization by nonconservative homologous recombination in XP-F cells.

Our findings have important implications for the infectious cycle of HSV and other human herpesviruses. In addition, they raise the possibility of using HSV-1-mediated transient replication of recombination products as a model for recombination and double-strand-break repair in human cells.

MATERIALS AND METHODS

Cells and viruses. BHK-21 cells (clone 13, ATCC CCL 10) were grown in Glasgow modified Eagle's medium (GIBCO) supplemented with 10% tryptose phosphate broth and 10% newborn calf serum (GIBCO) at 37°C in an atmosphere of 5% CO_2. HSV-1, Glasgow strain 17 syn^+, was propagated in the cells under the conditions specified above. The viral stocks were prepared and the virus titer was measured as described previously (11).

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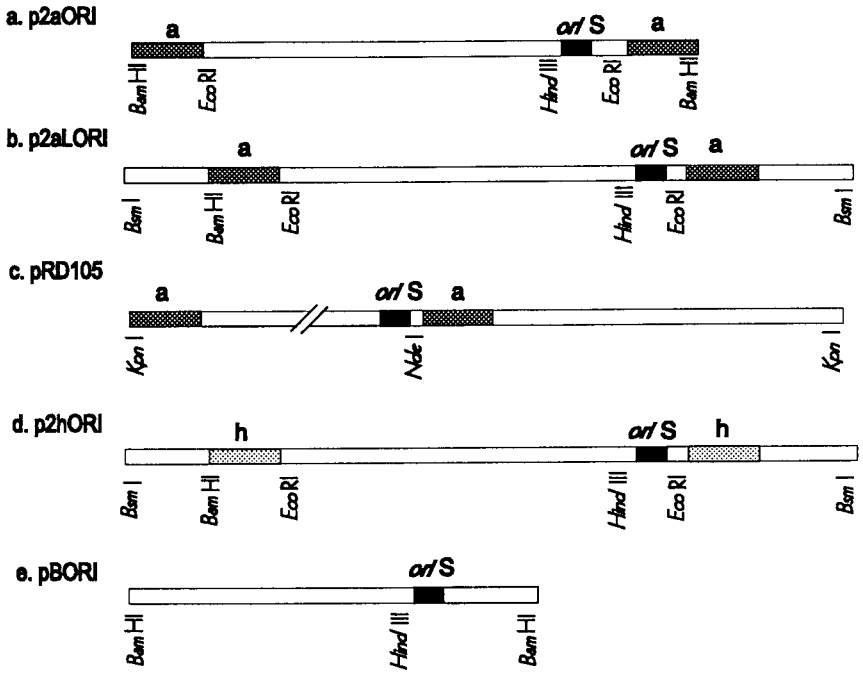

FIG. 1. Structures of plasmids p2aORI, pBORI, p2aLORI, p2hLori, and pRD105. *Bam*HI, *Bsm*I, or *Kpn*I was used to linearize plasmid DNA before transfection. *Hin*dIII or *Nhe*I was used to produce plasmid monomers from replicated concatemers. *Eco*RI was used to identify *a*-sequence dimers and monomers.

Human XP-F cells (NIGMS Human Genetic Mutant Cell Repository [Camden, N.J.] GM08437A) and human repair-proficient ECACC 1BR.3N (hereafter called 1BR.3N) cells were grown in Eagle modified essential medium supplemented with 2 mM glutamine, 1% nonessential amino acids, and 10% fetal bovine serum at 5% CO_2.

Plasmids and bacteria. Plasmid pRD105 (5), containing the end element of the HSV-1 genome (the *a* sequence), was a generous gift from R. E. Dutch, Department of Biochemistry, Stanford University. Plasmids pE5, pE8, pE9, pE29, pE30, pE42, and pE52 were kindly provided by Nigel D. Stow, MRC Virology Unit, Institute of Virology, Glasgow, United Kingdom (32). These plasmids contain the seven essential genes required for HSV-1 replication. Expression of the viral gene products is controlled by the major human cytomegalovirus immediate-early promoter.

Four new plasmids were constructed for the recombination and replication experiments. Plasmid pUC19 was used as the parent vector for all constructions. First, the *a* sequence from HSV-1 strain KOS with *Bam*HI linkers on each end was removed from pRD105. The corresponding fragment, which is 317 bp in length, contained the DR1-Ub-$(DR2)_{10}$-Uc-DR1 element sequence. It was ligated to form the *a*-sequence dimer. Blunt ends were introduced by filling in the ends with the large fragment of DNA polymerase I. The *a*-sequence dimer was ligated to previous *Bam*HI site of pUC19 by blunt-end ligation after treatment of the vector with the large fragment of DNA polymerase I as described above. *Escherichia coli* SURE cells (Stratagene) were transfected, and a plasmid, p2a, containing an *a*-sequence dimer was identified. Second, a fragment containing HSV-1 oriS and the T7 promoter with *Pst*I/*Eco*RI and *Xba*I/*Hin*dIII adapters on the ends was cloned at the *Pst*I and *Hin*dIII sites of plasmid p2a. The resulting plasmid was designated p2aORI. It can be linearized by *Bam*HI to produce a double-stranded linear molecule with the *a* sequence as direct repeats at the ends (Fig. 1).

Plasmid p2aLORI was constructed by inserting a fragment from bacteriophage lambda between the directly repeated *a* sequences. The plasmid could be linearized by *Bsm*I cleavage in the lambda sequence.

A plasmid with direct repeats of a nonviral sequence was derived from p2aORI by cloning a 161-bp *Bam*HI fragment dimer from DNA of human fibroblasts. The *a*-sequence dimer on the *Eco*RI sites of p2aORI was replaced by an *Eco*RI/*Bgl*II/*Eco*RI adapter. It was then cleaved with *Bgl*II and ligated with the dimer from human DNA. The lambda fragment from pRD105 was then introduced at the *Bam*HI site separating the direct repeats. This plasmid is referred to as p2hLORI.

A control plasmid, pBORI, was obtained by ligating a fragment containing HSV-1 oriS and the T7 promoter to the pUC19 *Bam*HI site as described above. Then the *Bam*HI sites were destroyed after limited cleavage with *Bam*HI, filling in of the ends, and blunt-end ligation. Finally, an oligonucleotide was inserted on the *Aat*II site of the plasmid, introducing a *Bam*HI site at a distance from oriS (Fig. 1).

Transient replication assays. The topological requirements were analyzed by using circular supercoiled and relaxed molecules isolated by agarose electrophoresis in the absence of ethidium bromide. Relaxed plasmid DNA was produced by treatment with wheat germ topoisomerase I. It was recovered from the agarose gels by using a DEAE-containing membrane (27). The samples of supercoiled, relaxed, and linear DNA were analyzed by agarose gel electrophoresis in the presence and absence of ethidium bromide in order to ensure the integrity and purity of the DNA.

Confluent monolayers with 4.6×10^5 cells in 15-mm wells were transfected, using liposomes, with circular and linear plasmids (0.5 μg/well) and supplemented with 250 μl of OptiMEM (GIBCO) at 37°C for 4 h (11). Equal volumes (250 μl) of the growth medium were added, and the cells were superinfected with wild-type virus at 8 PFU per cell. Incubation was for 17 h. The medium was removed, and the cells were lysed in 550 μl of cell lysis buffer (1.2% sodium dodecyl sulfate, 50 mM Tris-HCl [pH 8.0], 4 mM EDTA, 4 mM $CaCl_2$, 0.2 mg of proteinase K per ml, 0.01 mg of calf thymus DNA per ml) at 37°C for 2 h. Total DNA was prepared by phenol extraction and ethanol precipitation. The DNA pellet was dissolved in 50 μl of double-distilled H_2O, and DNA was cleaved overnight with restriction endonucleases as indicated below. The cleaved DNA was subjected to electrophoresis on 1% agarose gels and subsequently transferred to Hybond-H^+ membrane by an alkaline blotting procedure (Amersham). The membrane was hybridized by using randomly primed pUC19 as a probe (Megaprime; Amersham). The amount of radioactivity of replicated DNA on the Southern blot was measured with a PhosphorImager (Molecular Dynamics).

In one series of experiments, virus was replaced with a series of plasmids containing all seven essential HSV-1 replication genes. The cells were cotransfected with the oriS-containing indicator plasmids and 0.2 μg of each of the seven plasmids. Incubation was for 30 h. Replicated DNA was analyzed as described above.

Structure of replication products. The extracted DNA from superinfected cells was cleaved with *Dpn*I, which degraded methylated bacterial input DNA. High-molecular-weight DNA consisting of *Dpn*I-resistant concatemers was isolated by electrophoresis in 0.7% SeaPlaque agarose gels. It was identified as the only prominent band in the high-molecular-weight region of the gel. DNA was recovered by phenol extraction and ethanol precipitation. The samples were cleaved with *Eco*RI and subjected to agarose electrophoresis. Following Southern blotting, the membranes were hybridized to a randomly primed *a*-sequence probe as described above. Restriction fragments from p2aORI cleaved with *Eco*RI and *Eco*RI/*Bam*HI, respectively, corresponding to *a*-sequence monomers and dimers were run as markers.

The structure of replicated DNA from experiments using linear p2aORI was

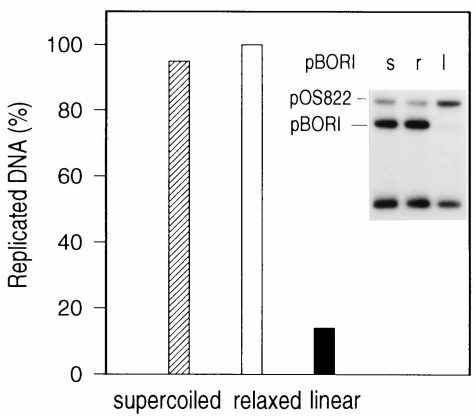

FIG. 2. Replication of different topological forms of plasmids in BHK cells. Circular supercoiled, relaxed, and linear pBORI DNA was used to transfect BHK cells. pOS822 was included as an internal standard. *Dpn*I- and *Hin*dIII-cleaved DNA was analyzed by Southern blotting of agarose gels. The histogram shows the efficiency of replication as measured with a PhosphorImager. (Inset) Autoradiograph of the samples used for quantitation. s, r, and 1 denote the supercoiled, relaxed, and linear forms of pBORI.

analyzed after transfection of *E. coli* with circularized replicated plasmid monomers. First, plasmid DNA was isolated from superfected cells by Hirt extraction (12) and cleaved with *Hin*dIII and *Dpn*I. The cleaved replication products were subjected to electrophoresis on 1% agarose gels. Plasmid monomers were recovered by using DEAE-containing membranes and circularized by using T4 DNA ligase. *E. coli* SURE cells were transformed with the ligation mixture. From each experiment, a number of colonies were selected and grown overnight in LB. Plasmid DNA was purified with a Wizard Mini-prep kit (Promega) and analyzed by restriction endonuclease cleavage.

Analysis of defective viruses. Confluent monolayers with 9×10^5 BHK cells (21-mm wells) were transfected using liposomes, with plasmid p2aORI (Fig. 1). The cells were superinfected with HSV-1 at 4 PFU per cell. Incubation was for 36 h. The cells, still in medium, were subjected to four cycles of freezing and thawing. The suspension was centrifuged at 2,000 rpm twice for 10 min each time at 4°C. This supernatant was defined as the first passage of defective virus. A second series of monolayers was infected with the first passage of virus (0.3 ml/well) for 36 h. The medium was removed. The monolayers were carefully washed with phosphate-buffered saline. They were frozen and thawed four times in phosphate-buffered saline. After centrifugation, the supernatant was supplemented with 1/3 volume of 20% polyethylene glycol 8000–2.5 M NaCl and incubated at 0°C for 10 min. The precipitate was collected by centrifugation at $10,000 \times g$ 30 min at 4°C. This pellet now contained virus particles from the second passage. The virions were incubated in the cell lysis buffer described above at 37°C for 1 h. DNA was prepared by phenol extraction and ethanol precipitation. DNA from the replicated defective virus was subjected to restriction enzyme cleavage and analyzed by Southern blotting and hybridization to either pUC 19 or *a*-sequence probes. The same procedure was performed with virus from the second passage.

RESULTS

Topological requirements for HSV-1-mediated replication of oriS-containing plasmids. We initially studied the topological requirements for efficient transient replication of oriS-containing plasmids in BHK cells superinfected with HSV-1. Circular supercoiled and relaxed plasmid DNA, pBORI, was used to transfect BHK cells (Fig. 2). The samples were examined by gel electrophoresis prior to the experiment to make sure that the amount of nicked DNA was negligible (results not shown). The ability of linear pBORI DNA to support DNA replication was investigated in the same way. In the transfection experiments, a constant small amount of the efficiently replicating plasmid pOS822 was included as an internal standard (9, 40). After the experiment, the DNA samples were cleaved with *Dpn*I and *Hin*dIII. Replicated DNA is resistant to cleavage with *Dpn*I. The upper bands on the autoradiographs therefore show replicated DNA, and the bands in the lower part of the autoradiographs correspond to fragments of input DNA generated by *Dpn*I. Our results revealed that circular supercoiled DNA and relaxed DNA were replicated with similar efficiencies. In contrast, linear pBORI DNA was replicated poorly (Fig. 2). The results suggest that HSV-1 DNA replication does not strictly depend on negative global supercoiling of the template prior to initiation of DNA synthesis. They also suggest that circularization of linear molecules is a prerequisite for amplification of template DNA by the HSV-1 replication apparatus.

Directly repeated *a* sequences enhance the replication of linear DNA. Since the HSV-1 genome is a double-stranded linear DNA molecule, we assumed the existence in the infected cells of mechanisms that stimulate the circularization and subsequent amplification of these molecules. Specifically, we wanted to see if the viral *a* sequence could stimulate the replication of linear DNA. Two series of plasmids were used. pBORI contains oriS and a single *Bam*HI site replacing the *Aat*II site in the pUC vector (Fig. 1). p2aORI contains two directly repeated *a* sequences separated by a *Bam*HI site. Linear DNA was produced by cleavage with *Bam*HI (Fig. 1). Circular or linear DNA was introduced by lipofection into BHK cells, and the cells were superinfected with HSV-1. We then isolated DNA at 17 h postinfection and subjected it to cleavage with *Dpn*I and *Hin*dIII. The replication products were analyzed by Southern blotting. The results showed that the replication of circular molecules was not affected by the *a* sequences. However, the directly repeated *a* sequences greatly stimulated the replication of linear DNA (Fig. 3).

Circularization of linear DNA proceeds either by end joining or by nonconservative homologous recombination. To examine the mechanism responsible for the efficient replication of linear DNA occurring in the presence of directly repeated *a* sequences, we examined the structures of the replication products. When the *Dpn*I-resistant DNA was subjected to cleavage with *Hin*dIII and *Bam*HI, we noted that *Bam*HI failed to cleave the replication products formed from linear DNA (Fig. 4). We therefore conclude that the linear molecules were not circularized by simple ligation.

We also noted that the replication of linear DNA formed a discrete product in the presence of directly repeated *a* sequences but gave rise to a smear in their absence (Fig. 4). Furthermore, in the presence of directly repeated *a* sequences, the replication products appeared to be shorter than the products obtained from the corresponding circular DNA (Fig. 4). A closer examination of the replication products was carried out in the following way. Large *Dpn*I-resistant concatemers were isolated by electrophoresis in low-melting-point agarose. Following phenol extraction, the isolated DNA was exposed to *Eco*RI, which cleaves on both sides of the *a*-sequence dimer in plasmid p2aORI (Fig. 1). The cleavage products were analyzed on agarose gels by using an *a*-sequence-specific probe. We found that the circular plasmid p2aORI gave rise to replication products containing the *a*-sequence dimer present at the outset of the experiment (Fig. 5). In contrast, linear p2aORI DNA gave rise to products containing either an *a*-sequence dimer or an *a*-sequence monomer (Fig. 5). The band corresponding to the *a*-sequence dimer was smeared, indicating sequence heterogeneity. We tentatively concluded that linear molecules containing directly repeated *a* sequences were efficiently circularized and subsequently amplified by the HSV-1 machinery. Two mechanisms appeared to be in operation: (i) end joining leading to an imprecise fusion of the *a* sequences and (ii) homologous recombination resulting in the formation of a unique product containing an *a*-sequence monomer.

To further examine the structure of the replication products,

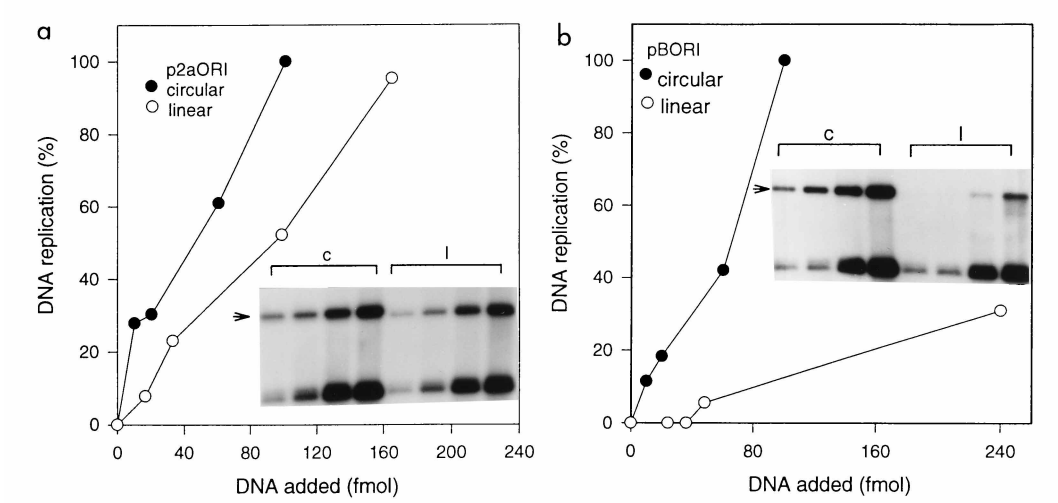

FIG. 3. Replication of circular and linear DNA with and without directly repeated *a* sequences. (a) The inset is an autoradiograph of a Southern blot showing *Dpn*I and *Hin*dIII cleaved DNA from an experiment with p2aORI. The *Dpn*I-resistant replication products are indicated by an arrow. In the lower part of the gel, the *Dpn*I fragments from input DNA can be seen. The graph shows the amounts of replication product obtained for the different forms of the plasmid as measured by a PhosphorImager. (b) DNA replication supported by circular and linear pBORI. The results are presented as described above.

we amplified the recircularized replication products in *E. coli*. Plasmid DNAs isolated from bacterial colonies were used to analyze the structures of replication products. Circular p2aORI gave rise to replication products that in 90% of the cases were identical with the parental plasmid (results not shown). Plasmids with a single *a* sequence were also seen at a low frequency. In contrast, when linear p2aORI was used, 61% of the plasmids isolated from *E. coli* contained only a single copy of the *a* sequence (results not shown).

We therefore suggest that homologous recombination can efficiently convert linear DNA containing directly repeated *a* sequences to circular templates for further amplification. In the absence of direct repeats, considerably less efficient end joining occurs, giving rise to a wider variety of circular products.

Linear DNAs containing directly repeated *a* sequences give rise to defective viruses. We wanted to make sure that the *a* sequence used in this series of experiments retained its biologically relevant functions of supporting the cleavage and packaging of virus particles. We therefore propagated replication products as defective viruses over three passages. The structures of defective viruses derived from the linear plasmids were examined by Southern blotting. The results showed that the circular plasmid p2aORI gave rise to products retaining the *a*-sequence dimer (Fig. 6, lane 6), whereas the linear version of the same plasmid predominantly produced *a*-sequence monomers (lane 7). Our results argue that the *a* sequence has retained its essential biological properties. They also emphasize that homologous recombination is an efficient way of producing templates for viral amplification.

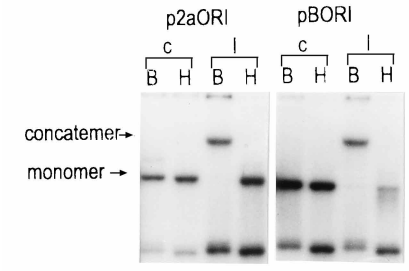

FIG. 4. Replication products formed from linear DNAs are resistant to *Bam*HI cleavage. The autoradiograph represents an experiment where circular (c) and linear (l) p2aORI and pBORI were used to transfect BHK cells. The cells were superinfected by HSV-1. Isolated DNA was cleaved with *Dpn*I and either *Hin*dIII (H) or *Bam*HI (B). Arrows show the positions of the *Dpn*I-resistant concatemers and monomers.

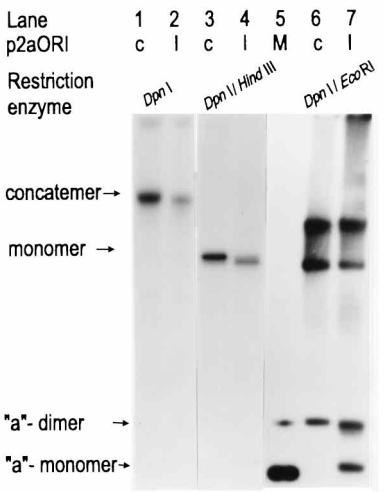

FIG. 5. Dimers and monomers of the *a* sequence can be found in the replication products. Circular (c) and linear (l) p2aORI was transfected into BHK cells. The cells were superinfected with HSV-1. The autoradiographs of Southern blots show the following. Concatemeric DNA was isolated by agarose gel electrophoresis (lanes 1 and 2). The concatemers could be cleaved to monomers by *Hin*dIII (lanes 3 and 4). Cleavage of the isolated concatemeric DNA with *Eco*RI released *a*-sequence dimers and monomers (lanes 6 and 7). A pUC probe was used for lanes 1 through 4; an *a*-sequence-specific probe was used for lanes 5 through 7. The upper bands in lanes 6 and 7 correspond to virus DNA. The positions of *a*-sequence dimers and monomers obtained from replicated plasmid DNA in the lower part of the autoradiograph are indicated. M (lane 5) denotes a marker sample.

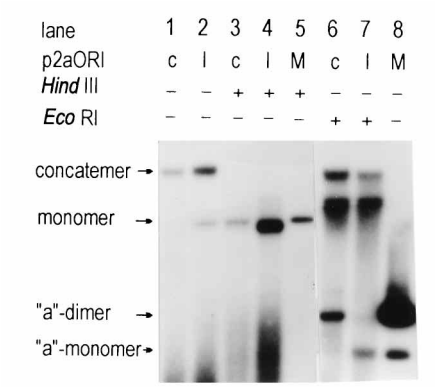

FIG. 6. Structures of defective viruses formed from circular and linear p2aORI DNA. Circular (c) and linear (l) p2aORI was transfected into BHK cells. The cells were superinfected with HSV-1. The autoradiographs of Southern blots show the following. Concatemeric DNA was isolated from virus particles after three passages (lanes 1 and 2). Monomers were released from concatemeric DNA by *Hin*dIII cleavage (lanes 3 and 4). Dimers and monomers of the *a* sequence were produced by cleavage of the isolated DNA by *Eco*RI (lanes 6 and 7). Markers (M) were linear p2aORI (lane 5) or a mixture of *a*-sequence monomers and dimers (lane 8). A pUC probe was used for lanes 1 through 5; an *a*-sequence-specific probe was used for lanes 6 through 8.

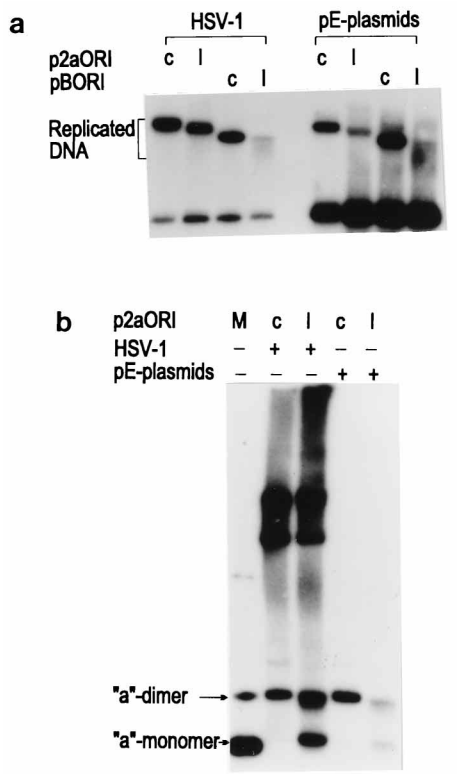

FIG. 7. Plasmids encoding the seven viral replication genes support replication of linear DNA containing directly repeated *a* sequences. Circular (c) and linear (l) p2aORI and pBORI were used to transfect BHK cells. The cells were either superinfected with HSV-1 or cotransfected with the pE series of expression plasmids. The autoradiographs show Southern blots of replicated DNA analyzed on agarose gels. (a) DNA was cleaved by *Dpn*I and *Hin*dIII. The position of replicated *Dpn*I-resistant DNA is shown. A pUC probe was used. (b) *Dpn*I-resistant concatemers were isolated and subsequently cleaved with *Eco*RI to analyze the structures of the *a* sequences as described for Fig. 6. An *a*-sequence-specific probe was used. The positions of *a*-sequence dimers and monomers are indicated. The bands in the upper part correspond to *a* sequences in the viral genome. M denotes a marker for *a*-sequence dimers and monomers.

Amplification of recombination products is supported by the seven viral replication genes. As an initial attempt to identify the genetic requirement for efficient homologous recombination during HSV-1 infection, we performed an experiment in which the superinfecting virus was replaced by a collection of plasmids encoding the seven essential HSV-1 replication genes (32). We found that efficient replication of the linear version of plasmid p2aORI took place also in the absence of complete viral infection (Fig. 7). In the absence of directly repeated sequences at the ends (pBORI), very limited amounts of discrete replication products were seen (Fig. 7a). We also found that the *a*-sequence dimer was retained when circular DNA was used in the transfection experiment (Fig. 7b). The corresponding linear plasmid again gave rise to products containing *a*-sequence monomers and a more diffuse band at the position of the *a*-sequence dimer (Fig. 7b). Our results suggest that the seven viral proteins are involved in replication whereas cellular proteins mediate recombination.

Nonconservative homologous recombination is not dependent on the distance between the direct repeats. Using virus-dependent amplification of the recombination products as an assay, we characterized the influence of the sequence arrangement on the recombination reactions. We used, in addition to plasmid p2aORI, plasmids that contain sequences separating the directly repeated *a* sequences (Fig. 1). Plasmid p2aLORI contains a 800-bp lambda fragment. In plasmid pRD105, the *a* sequences are separated by 2,700 bp. The plasmids were linearized by restriction enzyme cleavage and used in the transient replication assay. The results showed that the circular DNAs mostly gave products that were identical with the starting plasmids (Fig. 8), along with a small amount of products that appear to have deletions of part of the plasmid. In contrast, the linear molecules predominantly gave rise to replication products that had deletions of one *a* sequence and the sequence separating the two direct repeats (Fig. 8). Approximately 5% of the replication products formed in experiments using circular plasmid molecules have undergone homologous recombination. Remarkably, approximately 90% of the replication products obtained in experiments using linear DNAs were formed by homologous recombination. It was also evident that efficient recombination did not require that the direct repeats be at the ends. In fact, they could be at least 400 bp away from the end and still support homologous recombination.

The HSV-1 *a* sequence promotes nonconservative homologous recombination in XP-F cells. The viral *a* sequence is involved in cleavage and packaging of viral genomes (23). It also takes part in the frequent inversion of the U_L and U_S parts of the genome that occurs during an infectious cycle (23). We have now constructed plasmids containing direct repeats from different sources in an attempt to examine the role of the DNA sequence in promoting nonconservative homologous recombination. One such plasmid, p2hLORI, contained a directly repeated restriction fragment of 161 bp from human fibroblasts. This plasmid and p2aLORI were then used to transfect three different cell lines: BHK-21, XP-F, and 1BR.3N. The XP-F and 1BR.3N cells were chosen to evaluate the role in recombination of the human homolog of Rad1 from *S. cerevisiae*, XPF/ERCC4 (29a, 41).

It was observed that circular molecules predominantly gave rise to replication products that were identical to the plasmid used to transfect the cells (Fig. 9). Interestingly, a small amount (between 5 and 10%) of a deletion product was also found. This product was seen in experiments using p2aLORI

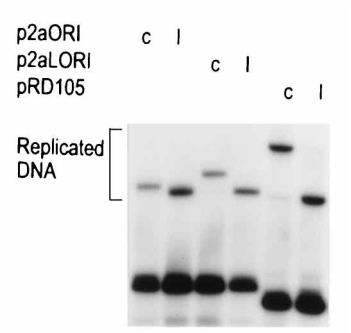

FIG. 8. Nonconservative homologous recombination does not depend on the distance between the directly repeated *a* sequences. Circular (c) and linear (l) p2aORI, p2aLORI, and pRD105 were used to transfect BHK cells. The cells were superinfected with HSV-1. DNA samples from transfection experiments with p2aORI and p2aLORI were cleaved with *Dpn*I and *Hin*dIII. DNA samples from experiments using pRD105 were cleaved with *Dpn*I and *Nhe*I. DNA was then analyzed by Southern blotting using a pUC-specific probe. The approximate positions of the amplified recombination products are indicated. Note that the linear DNA predominantly support the formation of a unique deletion product.

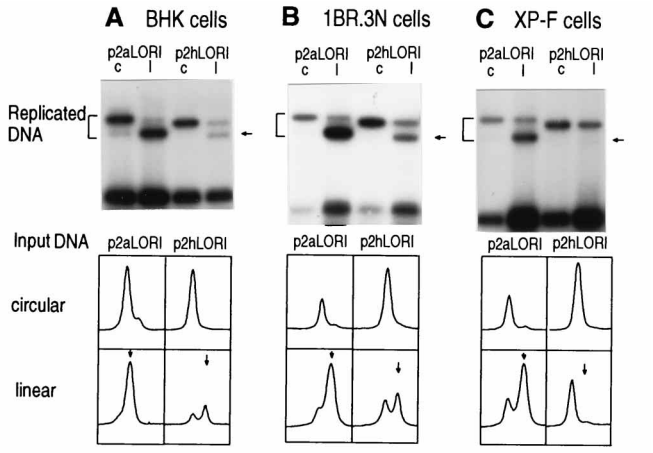

FIG. 9. The HSV-1 *a* sequence promotes efficient nonconservative homologous recombination in XP-F cells. Circular (c) and linear (l) p2aLORI and p2hLORI were transfected into BHK cells, repair-proficient human 1BR.3N cells, and XP-F cells, which were then superinfected with HSV-1. The autoradiographs show Southern blots of *Dpn*I- and *Hin*dIII-cleaved replicated DNA. The arrows show the positions of the product formed by nonconservative homologous recombination. The autoradiographs were scanned with a Molecular Dynamics densitometer, and the results are shown in separate panels. Arrows show the positions of the deletion products.

but not in experiments using p2hLORI. Similar results were obtained for all cell lines used. These results agree with the observation that homologous recombination in mammalian cells depends on the length of the homologous sequences (2, 26).

Linear DNAs from plasmids p2hLORI and p2aLORI were also used in the same way (Fig. 9). We found that linear p2aLORI replicated well in all cells. More than 90% of the replication products were formed by nonconservative homologous recombination, thereby deleting one *a* sequence and the separating lambda sequence. Plasmid p2hLORI replicated in all cells. However, in BHK and 1BR.3N cells, only approximately 50% of the replicated DNA corresponded to the deletion product. More importantly, in XP-F cells, only small amounts of the deletion product was formed with linear p2hLORI (Fig. 9). We also examined recombination between nonviral repeats of different lengths. We found that repeated sequences of 341 and 800 bp also promoted nonconservative homologous recombination in XP-F cells. The 341-bp sequence was, however, less efficient in this respect than the 317-bp *a* sequence (results not shown).

We interpret our results in the following way. General homologous recombination gives rise to deletions in circular molecules at a low but significant frequency. The reaction is characterized by its dependence on the length of the homologous sequences. The enzymatic machinery might be virus specific or belong to the RAD52 epistasis group as defined for *S. cerevisiae* (21, 34). The frequency of this reaction was in our hands the same as that in previously published experiments (5). Nonconservative homologous recombination and imprecise end joining operate on linear molecules. At least two distinctly different pathways for nonconservative homologous recombination seem to exist.

One pathway, commonly referred to as single-strand annealing, appears to be dependent on XPF/ERCC4 (6, 13, 17, 18). This reaction, however, did not seem to be required for circularization of p2aLORI and viral genomes. In fact, linear DNAs containing repeated *a* sequences were efficiently circularized in XP-F cells. Furthermore, the efficiency of viral DNA replication was not reduced in these cells (results not shown).

The other pathway may depend on the length of the homologous sequences and involve the mammalian counterpart to the RAD52 epistasis group. Our results indicate that this pathway operated in all cells and promoted efficient circularization of linear p2aLORI. In contrast, linear p2hLORI containing a shorter repeated sequence was not circularized efficiently by this pathway.

DISCUSSION

Three different pathways can join DNA ends in cells infected with HSV-1. We have used the efficient replication apparatus from HSV-1 to amplify oriS-containing plasmids in mammalian cells. We found that also linear DNA molecules could be replicated, but that the replication products were derived from molecules that had taken part in different recombination reactions. Two distinct products of recombination were seen. In the first instance, the ends of linear DNA were fused by an imprecise end-joining mechanism and not by simple ligation. This reaction was probably the same as the most frequently used way to repair double-strand breaks in mammalian chromosomes (8, 24, 25, 38). In the second case, the ends were joined by nonconservative homologous recombination requiring the presence of directly repeated sequences (10). In uninfected mammalian cells, nonconservative homologous recombination is thought to occur by a single-strand annealing mechanism (17, 18). In *S. cerevisiae*, this reaction is dependent on Rad1/Rad10 endonuclease (6, 13).

Our results revealed that linear DNA containing direct repeats of a 161-bp sequence of nonviral origin was circularized efficiently by nonconservative homologous recombination in repair-proficient 1BR.3N cells but not in XP-F cells. This result indicates that ERCC4, a mammalian homolog of the Rad1 endonuclease, is a component of the single-strand annealing pathway in mammalian cells. A completely different result was obtained when linear DNA molecules containing directly repeated viral *a* sequences were used. These molecules were readily circularized by nonconservative homologous recombination also in XP-F cells. Additional mechanisms that promote circularization of linear DNA might therefore exist. An apparent alternative would be homologous recombination catalyzed by members of the RAD52 epistasis group as defined for *S.*

cerevisiae (21). Recombination between direct repeats of the viral *a* sequence appears to use this pathway. The reaction is not sequence specific since different long direct repeats of nonviral origin also promote circularization of linear DNA in XP-F cells (42). Our results do not, however, exclude that the *a* sequence may enhance the frequency of homologous recombination. For instance, it may provide double-strand breaks in a UL6-dependent way or increase the frequency of strand breaks occurring during DNA replication (15, 30).

Mechanisms for circularization of the HSV genome. Herpesviruses all have linear genomes of double-stranded DNA that need to be circularized before efficient replication of the viral chromosomes can take place (23). Circularization has been shown to be an early step in the infectious cycle. At least in some instances, it may occur in the presence of cycloheximide (7, 22). It has, however, not been shown that the reaction that proceeds in the presence of inhibitors is the pathway followed during an undisturbed infectious cycle. What is the mechanism of circularization? Viral genomes usually contain at their 3′ termini unpaired complementary single nucleotides produced by the viral machinery for cleavage and packaging (20). Direct ligation of ends may therefore occur, and the reaction might be promoted by proteins that assist in bringing the ends together. These proteins remain undefined. An alternative or complementary mechanism would be homologous recombination. In its simplest version, a processive exonuclease might produce single-stranded tails that could anneal (36). This reaction calls for a complementing reaction by which single terminal repeats can be reiterated. Mechanisms for such reactions have been proposed (20, 23, 37). An inherent advantage of using homologous recombination as a method for circularizing chromosomes is that the process would become less sensitive to accidental shortening of the end sequences.

The experiments in this study describe circularization of linear DNA containing directly repeated HSV-1 *a* sequences. The results suggest that homologous recombination may serve as an efficient way to circularize viral genomes and to repair double-strand breaks in human cells. The mechanism of the reaction is not defined, but one might speculate that single-strand-initiated homologous recombination occurs. It has recently been found that the mammalian homolog of *S. cerevisiae* Rad51 is essential for growth of mammalian cells (16, 35). This observation suggests that the RAD52 epistasis group operates also in human cells (21, 34). We are currently trying to express mutant forms of the human Rad51 protein to inhibit this pathway (3). Such experiments will hopefully clarify the contributions of cellular and viral proteins to homologous recombination in HSV-1-infected cells (1). The genome of HSV-1 also takes part in a second recombination reaction. The L and S components of the HSV-1 genome invert relative to each other during a normal replication cycle. The *a* sequence is dispensable for this reaction (19). Only an extended region of sequence homology is required. It is an interesting possibility that circularization of genomes and inversion of the L and S segments use the same mechanism. In fact, the double-strand-break repair model for recombination would predict this (31, 33). It is worth noting that the number of *a* sequences would be conserved under these circumstances.

Concluding remarks. In summary, in this report we show that linear DNA containing directly repeated sequences can be converted by nonconservative homologous recombination to templates for replication by the HSV-1 replication apparatus. The reaction appears not be sequence specific, but it depends on the length of the homologous sequences. The HSV-1 *a* sequence efficiently utilizes this pathway. We now propose that viral genomes might be circularized by homologous recombination. It is, however, important to keep in mind that direct ligation of genomic termini remains a plausible alternative. A genetic analysis is clearly needed to resolve this issue. Other interesting questions that could be addressed by using HSV-1-mediated amplification of recombination products concern the factors and conditions that determine the choice between the different recombination pathways in human cells.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Cancer Society (2552-B95-09XBC), the Medical Research Council (K97-13X-12199-01A), the Åke Wiberg Foundation, and the Wilhelm and Martina Lundgren Fund.

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