Homologous recombination was examined in cells infected with herpes simplex virus type I. Circular and linear DNA with directly repeated sequences was introduced as recombination substrates into cells. Recombination was measured either by origin-dependent amplification of recombination products or by recombination-dependent expression of luciferase from a disrupted gene. Homologous recombination in baby hamster kidney cells converted linear DNA to circular templates for DNA replication and luciferase expression in the complete absence of virus. The products of homologous recombination were efficiently amplified by the viral replication apparatus. The efficiency of recombination was dependent on the structure of the substrate as well as the cell type. Linear DNA with the direct repeats at internal positions failed to recombine in Balb/c 3T3 cells and induced p53-dependent apoptosis. In contrast, linear DNA with directly repeated sequences precisely at the ends recombined and replicated in 3T3 cells. Homologous recombination in baby hamster kidney cells did not depend on the position of the repeated sequences. We conclude that homologous recombination is independent of viral gene functions and that it is likely to be carried out by cellular proteins. We suggest that homologous recombination between directly repeated sequences in the linear herpes simplex virus type I chromosome may help to avoid p53-dependent apoptosis and to promote viral DNA replication.

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‡ The abbreviations used are: HSV-1, herpes simplex virus type I; XP-F, xeroderma pigmentosum complementation group F; MEF, mouse embryonic fibroblast; BHK, baby hamster kidney; bp, base pair(s); CMV, cytomegalovirus.

constitute one way to promote circularization of linear viral genomes containing extensive directly repeated sequences at the ends (7). A recent study, on the other hand, demonstrates that circularization of the guinea pig cytomegalovirus genome is likely to proceed by a different mechanism (8). So far, no gene products, viral or cellular, have been identified that directly take part in the circularization of viral genomes.

Three distinct pathways involved in the repair of double-stranded breaks have been detected in eukaryotic cells. One of these, referred to as nonhomologous end joining, involves Ku and the DNA-dependent protein kinase (9). Inside cells, it appears to yield products that contain an altered DNA sequence at the site of fusion. More recently, an in vitro system that depends on DNA ligase IV/Xrc4 and requires Ku70, Ku86, and the catalytic subunit of DNA-dependent protein kinase has been developed. This system produces accurate and efficient ligation of complementary ends (10).

A second alternative is nonconservative homologous recombination using a single strand annealing mechanism. In Saccharomyces cerevisiae, an endonuclease formed by the rad10 and rad1 gene products is strictly required (11). The mammalian homologues are the ERCC1 and ERCC4 gene products. The loss of the ERCC1 gene in mammalian cells results in an increase of recombination-dependent deletions at tandemly repeated sequences and an increased instability of genomes (12, 13). XP-F cells that are devoid of the ERCC4 gene product show an impaired capacity to circularize linear DNA molecules with short direct repeats, but they can efficiently recombine long homologous sequences (14).

The third pathway is homologous recombination, which in S. cerevisiae depends on the rad51 gene product and the other members of the rad52 epistasis group (15). The mammalian homologue, HsRad51, appears to be essential for viability of cells (16, 17). It is believed to act primarily in recombination repair during the late S-G2 phase of the cell cycle (18). Over-expression of Rad51 protein increases the frequency of recombination events and the resistance of mammalian cells to ionizing radiation (19). It has been observed that homologous recombination in mammalian cells is dependent on the length of the homologous regions (14, 20, 21).

Ancient viruses that establish life-long infections in the host are likely to interact with the cellular pathways that control the replication and integrity of cellular chromosomes. We have performed a series of investigations aimed at identifying interactions between the DNA metabolism of herpes simplex virus type I and the infected cell. For example, we found that inhibition of topoisomerase II by ICRF-193 prevented efficient replication of HSV-1, indicating that decatenation of replication products was an early and essential step during virus replication (22). We also noted that direct repeats of the HSV-1 terminal sequence were able to promote nonconservative homol-
ogous recombination not dependent on XP-F/ERCC4 (14). In fact, linear DNA molecules containing the HSV-1 origin of replication, oriS, as well as directly repeated sequences replicated as efficiently as the corresponding circular molecule in BHK cells (14).

We have now investigated if viral DNA synthesis or viral gene products are required for recombination to occur. We have also looked at the efficiency of homologous recombination in different cell types. A new assay that allowed us to monitor homologous recombination in the absence of HSV-1 DNA replication was developed. Briefly, a plasmid that supports the expression of luciferase was redesigned to contain the middle portion of the coding sequence as direct repeats. The repeated sequences were separated by a noncoding sequence derived from bacteriophage λ. Linear plasmid DNA was produced by restriction enzyme cleavage in the λ sequence and introduced by lipofection into cells. The correct open reading frame could now be restored by homologous recombination. This assay, referred to as recombination-dependent expression of luciferase, was combined with a described previously assay for oriS-dependent amplification of recombination products to monitor how a HSV-1 infection and the genetic background of cells affect the efficiency of homologous recombination.

The results show that homologous recombination is mechanically independent of HSV-1 but dependent on the properties of the cell. Interestingly, the small amounts of recombination products that are formed are very efficiently amplified by the HSV-1 DNA synthesis machinery, suggesting a structural or temporal coupling between these processes.

MATERIALS AND METHODS

Cells and Viruses—BHK cells (clone 13 ATCC CCL 10) were grown in Glasgow modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% tryptose phosphate broth, 10% newborn calf serum, 50 units/ml penicillin, and 50 µg/ml streptomycin at 37 °C in an atmosphere of 5% CO₂. COS-7 (ATCC CCL 1651) cells and mouse embryo fibroblasts (MEFs) were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin. MEFs were from Maria Enge (Department of Medical Biochemistry, Göteborg University). Balb/c 3T3 cells (ATCC CLL 163) and Balb/c 3T3.SV-T2 cells (ATCC CCL 163–1) were grown in Dulbecco's modified Eagle's medium with 10% calf serum, 2 mM glutamine, and 4.5 g/liter glucose.

HSV-1 Glasgow strain 17 syn+ was propagated at 37 °C and the viral stocks were prepared as described previously (14, 22). HSV-1 tsK strain (kindly provided by Dr. Nigel Stow, MRC Virology Unit, Institute of Virology, Glasgow, United Kingdom). Titration of all viruses was performed on BHK cells.

Reagents—Phosphonoacetic acid, luciferin, and camptohecin were from Sigma.

Plasmids—A series of plasmids containing repeated sequences has been described (Fig. 1; Refs. 2 and 14). In some cases, the directly repeated sequences are separated by a spacer of A DNA derived from the plasmid pRDI05 (2, 14). They were designated p2aLORI, p2bLORI, and p2bLORI, depending on the source of the repeated sequences (Fig. 1A). The repeated sequences were the 317-bp a sequence from the HSV-1 genome, a 341-bp sequence from BHK cells, and a 161-bp sequence of human origin. Another set of plasmids without the λ DNA spacer, p2aORI and p2bORI, was described previously (14). The plasmid pBori lacks repeated sequences (14). The HSV-1 origin of replication, oriS, was included to allow transient replication in cells superinfected with HSV-1. Another series of plasmids, p2aLSV and p2hLSV, contains repeated sequences as well as the SV40 origin of replication.

A new series of plasmids was designed to measure recombination-dependent expression of luciferase (Fig. 1B). The functional gene was divided into three parts starting from the 5'-end and ending with the SV40 polyadenylation signal. They were called LucI (612 bp), LucII (776 bp), and LucIII (1222 bp). These sequences were cloned into an expression vector, pCMV19K, from which E1B19K and the SV40 origin were deleted (14). The HSV-1 oriS was cloned downstream of the luciferase gene. pXY2 contains the intact luciferase gene. In pXY2, LucI was duplicated, and the two copies were separated by a λ sequence. The restriction fragments Fr1, Fr2, and Fr3 contain only parts of the luciferase gene (Fig. 1). Fr1 and Fr2 were from pXY2. Fr3 was derived from a different plasmid. It contains LucII and LucIII but lacks the CMV promoter. The DNA molecules were used either as circular DNA or as linear DNA produced by restriction enzyme cleavage and used in Fig. 1.

Transfection Protocols—Circular or linear plasmid DNA was introduced into cells using liposomes. BHK and COS-7 cells were transfected as described previously (14, 22). 3T3 cells and MEFs were transfected using LipofectAMINE PLUS as described by the manufacturer (Life Technologies, Inc.). 0.25–1 µg of DNA was mixed with 50 µl of Optimem and 50 µl of Plus reagent. The mixture was incubated at room temperature for 15 min. Then 2 µl of LipofectAMINE and 50 µl of Opti-MEM were added. The 105-µl suspension of liposomes was used for the transfection of cells together with 400 µl of Opti-MEM in 21-mm dishes.

The frequency of transfection was determined using the plasmid pCMVβ (CLONTECH) encoding β-galactosidase. The transfected cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactoside. For each monolayer, the frequency of cells expressing β-galactosidase was determined at several locations in the wells. An average of the values obtained for each well was used to construct Table II.

Transient Replication Assay—Subconfluent monolayers of cells were transfected by circular or linear plasmid DNA using liposomes for 4 h. Supernatant with HSV-1 (5 pfu/cell) or cotransfection with a collection of expression plasmids was used to infect the cell monolayer. The transfection protocol described previously (24). Total DNA was harvested either 18 h after superinfection or 48 h following cotransfection with expression plasmids. DNA was digested with restriction enzymes as indicated and subjected to electrophoresis in 1% agarose gels. The enzyme DpnI was used to cleave input DNA. The DNA was cloned onto HYBOND-H+ membranes (Amersham Pharmacia Biotech) as described by the manufacturer. Hybridization was performed using radiolabeled probes (Megaprime; Amersham Pharmacia Biotech). This probe recognizes the oriS-containing plasmids as well as the pE series of expression plasmids. Probes from the fragments a, b, and h were used in the experiment shown in Fig. 2.

Expression of Luciferase-dependent on Recombination—The plasmid pXY2 was used as a substrate for recombination. The middle part of the luciferase gene, LucII, was present as direct repeats separated by a λ DNA sequence (Fig. 1B). Homologous recombination between the LucII repeats will create an intact luciferase gene, which can be monitored by a luciferase assay. Confluent or subconfluent monolayers of BHK cells in 15-mm dishes and Balb/c 3T3 or 3T3.SV-T2 cells in 21-mm dishes were lipofected as indicated. The cells were harvested after 24 h. The monolayers were washed twice with cold phosphate-buffered saline. They were suspended in 0.1 M potassium phosphate, pH 7.8, and centrifuged at 12,000 × g at 4 °C for 2 min. The cell pellet was resuspended in 50 µl of the same solution. This suspension was repeatedly frozen and thawed three times. The cell lysate was cleared by centrifugation at 12,000 × g at 4 °C for 5 min. Luciferase activity was measured by taking 20 µl of the supernatant mixed with 0.36 ml of a buffer containing 200 mM Tris-HCl, pH 6.8, 0.1 M dithiothreitol, 2% SDS, 0.1% bromphenol blue, and 10% glycerol and boiled for 3 min. The samples were loaded on a 10% SDS-polyacrylamide gel. Electrophoretic transfer was done onto Immobilon-P membrane (Millipore Corp.). The immunoreaction of the blotted membrane was performed by using Bio-Rad Immun-Blot assay kit (goat anti-rabbit IgG, alkaline phosphatase) and an anti-luciferase antibody (Clontech, Palo Alto, CA).

Assays for Apoptosis—Monolayers of cells in 21-mm dishes were transfected with liposomes containing either circular or linear forms of the plasmid pCMVβ as indicated. The transfected cells were incubated for 20 h and subsequently stained with 5-bromo-4-chloro-3-indolyl-β-D-galactoside as described by the manufacturer (CLONTECH). The total number of blue cells expressing β-galactosidase and the number of

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Apoptosis was also measured by staining cells with annexin V coupled to green fluorescent protein using the ApoAlert™ kit from CLONTECH. Balb/c 3T3 cells were grown on glass coverslips in 35-mm dishes. The cells were lipofected at 80% confluency with 1.6 g of circular or linear pCMVβ. The growth medium was collected at 8 h post-transfection, and the monolayers were rinsed three times with 1 ml of phosphate-buffered saline. The nonadherent cells were collected by centrifugation. The adherent and nonadherent cells were stained separately with annexin V-EFGP as described by the manufacturer. The cells were washed after staining. The nonadherent cells were suspended in 20 µl of phosphate-buffered glycerol prior to fluorescence microscopy.

RESULTS

Coupling of Homologous Recombination and DNA Replication—To study mechanisms of recombination in mammalian cells, we have developed a transient replication assay to monitor the circularization of linear DNA (14). We constructed plasmids that contained directly repeated sequences that in some instances were separated by a sequence of λ DNA (Fig. 1). The molecules formed by end joining are found in a slightly diffuse band in the upper part of the autoradiograph. The two bands in the middle of the autoradiograph represent linear input DNA. Imprecise nonhomologous end joining or homologous recombination produces different products (14). We initially examined the fate of transfected linear DNA in infected and uninfected BHK cells (Fig. 2). Total DNA was isolated from cells undergoing a transient replication experiment and cleaved with EcoRI prior to agarose gel electrophoresis. The two bands in the middle of the autoradiograph represent linear input DNA. Imprecise nonhomologous end joining or homologous recombination produces different products (14). We initially examined the fate of transfected linear DNA in infected and uninfected BHK cells (Fig. 2). Total DNA was isolated from cells undergoing a transient replication experiment and cleaved with EcoRI prior to agarose gel electrophoresis. The two bands in the middle of the autoradiograph represent linear input DNA. Imprecise nonhomologous end joining or homologous recombination produces different products (14).
Recombination-dependent Expression of Luciferase Does Not Require HSV-1—To further elucidate the interdependence of DNA replication and recombination, we devised an assay that allowed us to measure recombination in the absence of DNA synthesis. A recombination substrate, pXY2, was made in which a luciferase gene was altered to contain directly repeated sequences separated by a DNA spacer (Fig. 1B). Controls contained either an intact luciferase gene, pXY1, or different parts of the luciferase genes as in Fr1, Fr2, and Fr3 (Fig. 1B). First, we introduced linear DNA molecules into BHK cells (Fig. 4A). We found that none of the DNA fragments containing incomplete luciferase genes supported luciferase expression when transfected into BHK cells (Fig. 4A). The linear form of the plasmid pXY1 containing the complete gene readily supported luciferase expression. We also found that linear pXY2 containing the disrupted luciferase resulted in high level expression of luciferase in BHK cells (Fig. 4A). This demonstrates that homologous recombination efficiently can restore the proper open reading frame for luciferase expression.

Recombination-dependent expression of luciferase. Plasmid DNA or restriction fragments derived from plasmids were transfected into BHK cells. The cells were incubated for 24 h. A cell lysate was prepared, and luciferase activity and protein concentration were measured. A, 0.5 μg of linear DNA was transfected into BHK cells. pXY1 is the intact gene; pXY2 is the disrupted gene; and Fr1, Fr2, and Fr3 are restriction fragments corresponding to different parts of pXY2 (see “Materials and Methods”). B, increasing amounts of DNA were transfected into BHK cells, and the luciferase activity was measured. C, 0.5 μg of DNA was transfected into BHK cells. Cell lysates were prepared at 24 h after transfection and analyzed by SDS-polyacrylamide gel electrophoresis. Western blots were then prepared using an anti-luciferase antibody.

With circular and linear pXY1. Linear pXY2 also supported luciferase expression. Circular pXY2, on the other hand, gave rise to very little luciferase. Apparently, a double strand break...
greatly stimulated homologous recombination, leading to restoration of an intact luciferase gene. We could also verify using SDS gel electrophoresis and Western blotting with an anti-luciferase antibody that the molecular weights of luciferase enzyme produced from the plasmids pXY1 and pXY2 were identical (Fig. 4C). Moreover, similar amounts of luciferase were synthesized in cells transfected with linear pXY1 and pXY2 (Fig. 4C). Together, these results argue that homologous recombination is able to circularize linear DNA very efficiently in BHK cells.

It was noted in the previous paragraph that only a very small part of the transfected DNA was circularized by homologous recombination. To investigate how the cellular machineries for recombination act on our model substrates we have compared oriS-dependent amplification of linear and circular DNA substrates in different cell lines. In the first experiment, circular and linear p2aLORI, p2hLORI, and p2hLORI were cotransfected with a set of seven expression plasmids encoding the HSV-1 replication genes (Fig. 6). The results demonstrate that the circular plasmids replicated readily in BHK cells whereas linear DNA was also replicated efficiently in BHK cells. In contrast, linear DNA replicated very poorly in Balb/c 3T3 cells (Fig. 6). The products were as before produced either by end joining or by homologous recombination. We also noted that neither the transfected linear nor circular DNA was significantly degraded in 3T3 cells (Fig. 6). The dramatic reduction of homologous recombination observed in Balb/c 3T3 cells could be explained by apoptosis induced by the presence of linear DNA. It has been demonstrated that DNA damage activates p53 as a transcription factor in Balb/c 3T3 cells and induces cell cycle arrest or apoptosis (26–28). The role of p53 was therefore examined in Balb/c 3T3.SV-T2 cells containing the SV40 large T-antigen as well as MEFs that were either wild type or homozygous knock-outs for p53.

First, recombination-dependent expression of luciferase was used to look at recombination in BHK cells and Balb/c 3T3 cells. We found again that there was efficient expression of luciferase from linear pXY1 as well as pXY2 in BHK cells (Fig. 7A). However, recombination-dependent expression of luciferase from pXY2 was very low in Balb/c 3T3 cells (Fig. 7B). Importantly, luciferase expression from linear pXY1 was also reduced. These results agree with those obtained using oriS-dependent amplification of recombination products as the assay (Fig. 6). In Balb/c 3T3.SV-T2 cells containing the SV40 large T-antigen, the expression of luciferase from circular and linear pXY1 did not differ, but recombination-dependent expression of luciferase from pXY2 was still lower than observed in BHK cells, indicating that inhibition of p53 activity abolishes the negative effect of linear DNA and partially restores recombination (Fig. 7C). Mouse embryo fibroblasts devoid of p53 (MEFP53/−/−) readily supported recombination-dependent expression of luciferase (Fig. 7D). Wild type mouse embryo fibroblasts were also examined. In this instance, the relative levels of luciferase produced by circular and linear plasmids containing intact genes were 100 and 70%, respectively.  

the inhibitor of viral DNA synthesis, phosphonoacetate, did not inhibit recombination-dependent expression of luciferase (Fig. 5).

We also examined the effects of viral gene expression using HSV-1 tsK and tsS strains at the nonpermissive temperature. The results demonstrated that recombination-dependent expression of luciferase was not affected significantly by the lack of either functional ICP4 or functional UL9 origin-binding protein (Fig. 5B).

By monitoring recombination-dependent expression of luciferase, we can examine large variations in the efficiency of homologous recombination. The presence of a double strand break will enhance homologous recombination more than 10-fold (Fig. 4B). In a similar way, the induction of apoptosis by linear recombination substrates results in a dramatic decrease in luciferase expression (see below). The variations in expression of luciferase shown in Fig. 5 are at least 1 order of magnitude smaller than the effect caused by a double strand break, and we believe that they most likely are caused by nonspecific effects on transcription. Our results therefore suggest that homologous recombination does not require HSV-1 DNA replication and gene expression.

Homologous Recombination Is Controlled by the Cell—To investigate how the cellular machineries for recombination act on our model substrates we have compared oriS-dependent amplification of linear and circular DNA substrates in different cell lines. In the first experiment, circular and linear p2aLORI, p2hLORI, and p2hLORI were cotransfected with a set of seven expression plasmids encoding the HSV-1 replication genes (Fig. 6). The results demonstrate that the circular plasmids replicated readily in Balb/c 3T3 cells and BHK cells. Linear DNA was also replicated efficiently in BHK cells. In contrast, linear DNA replicated very poorly in Balb/c 3T3 cells (Fig. 6). The products were as before produced either by end joining or by homologous recombination. We also noted that neither the transfected linear nor circular DNA was significantly degraded in 3T3 cells (Fig. 6). The dramatic reduction of homologous recombination observed in Balb/c 3T3 cells could be explained by apoptosis induced by the presence of linear DNA. It has been demonstrated that DNA damage activates p53 as a transcription factor in Balb/c 3T3 cells and induces cell cycle arrest or apoptosis (26–28). The role of p53 was therefore examined using Balb/c 3T3.SV-T2 cells containing the SV40 large T-antigen as well as MEFs that were either wild type or homozygous knock-outs for p53.

Fig. 5. Effects of HSV-1-supported DNA replication on recombination-dependent expression of luciferase. Linear DNA (0.5 μg) was transfected into BHK cells. The cells were then superinfected with wild type or mutant strains of HSV-1 in the presence and absence of phosphonoacetate (PAA). Cell lysates were prepared, and the luciferase activity was measured. A, effects of virus replication on luciferase expression. B, the effect of mutant viruses at the nonpermissive temperature on luciferase expression. The tsK virus harbors a mutation in the ICP4 gene, and tsS virus has a mutation in the UL9 gene.

By monitoring recombination-dependent expression of luciferase, we can examine large variations in the efficiency of homologous recombination. The presence of a double strand break will enhance homologous recombination more than 10-fold (Fig. 4B). In a similar way, the induction of apoptosis by linear recombination substrates results in a dramatic decrease in luciferase expression (see below). The variations in expression of luciferase shown in Fig. 5 are at least 1 order of magnitude smaller than the effect caused by a double strand break, and we believe that they most likely are caused by nonspecific effects on transcription. Our results therefore suggest that homologous recombination does not require HSV-1 DNA replication and gene expression.
Luciferase produced by the linear disrupted gene was 25% when compared with the circular template (results not shown). Together, these results demonstrate that homologous recombination is tightly controlled by the cell. One mechanism might be induction of p53-dependent apoptosis by linear DNA.

Linear DNA Induces p53-dependent Apoptosis in 3T3 Cells—As mentioned above, DNA damage activates p53 as a transcription factor in Balb/c 3T3 cells and induces cell cycle arrest or apoptosis (26–28). DNA-dependent protein kinase or ATM-related protein kinases may be responsible for the activation of p53 (26, 27, 29–31). Protein kinases or other signaling devices may recognize DNA ends introduced into cells by transfection and thereby initiate a process leading either to cell cycle arrest or cell death (32). We now wanted to examine if p53 either had a direct effect on homologous recombination or exerted an indirect effect by promoting the induction of apoptosis by linear DNA.

The induction of apoptosis was first monitored by a morphological assay. We used plasmid DNA encoding β-galactosidase under the control of the HCMV promoter. Circular and linear forms of plasmid DNA were then transfected into cells, and the morphology of the cells expressing encoding β-galactosidase was registered after staining with 5-bromo-4-chloro-3-indolyl-β-d-galactoside. The appearance of shrunk blue cells with pyknotic nuclei was used as an indicator of apoptosis. The linear DNA was produced by ScaI cleavage 1.7-kilobase pair downstream the polyadenylation signal. We noted that linear DNA increased the frequency of apoptotic cells as defined by morphological criteria —4.4-fold for Balb/c 3T3 cells and wild type mouse embryo fibroblasts (Table I). BHK cells, Balb/c 3T3.SV-T2, and p53-deficient mouse embryo fibroblasts were not affected by linear DNA in the same way.

The induction of apoptosis by transfected linear DNA was also investigated by staining of Balb/c 3T3 cells with annexin V, which is an early indicator of apoptosis (33). First, we looked at adherent Balb/c 3T3 cells. We found that the proportion of adherent cells binding annexin V was dramatically reduced. In contrast, p53-deficient mouse embryo fibroblasts were not affected by linear DNA in the same way.

Finally, we examined the effect of circular and linear DNA on the transformation frequency. We found that circular plasmids readily transformed all cell lines (Table II). Experiments using linear DNA produced a different picture. The transfaction frequencies for Balb/c 3T3 cells and wild type mouse embryo fibroblasts were dramatically reduced. In contrast, p53-deficient mouse embryo fibroblasts and BHK cells were readily transformed by linear DNA.

Our results suggest that linear DNA molecules with nonhomologous end sequences induce p53-dependent apoptosis in Balb/c 3T3 cells. This finding may help to explain the low levels of recombination-dependent expression of luciferase observed in Balb/c 3T3 cells (Fig. 7B). It should be noted that BHK cells most probably express wild type p53 (34). Other factors therefore must have a decisive influence on the outcome of recombination reactions occurring in the cell.

The Structure of the Recombination Substrate Determines the Efficiency of Homologous Recombination in 3T3 Cells—Herpes viruses that introduce linear genomic DNA into mammalian
cells run the risk of triggering an apoptotic response. We have shown above that linear DNA unable to undergo homologous recombination induces apoptosis in Balb/c 3T3 cells (Table I). As a result, linear DNA molecules appear to replicate very poorly in nontransformed Balb/c 3T3 cells and fail to support recombination-dependent expression of luciferase (Figs. 6 and 7B). Cells appear to face a situation where a decision has to be made between different pathways of recombination repair as well as between different fates of the cell. The structure of the broken DNA molecule may influence this decision. We have for this reason compared the replication and recombination of the linearized plasmids p2aLORI, p2aORI, and p2bORI in Balb/c 3T3 cells and BHK cells (Fig. 8A). The linearized plasmids p2aORI and p2bORI have the repeated sequences at the ends of the DNA molecule, and they were circularized and replicated by the HSV-1 replication machinery in both cell types. The insertion of a λ DNA insert between the directly repeated λ DNA spacer, p2aLORI, or without a λ spacer, p2aORI and p2bORI, were transfected into BHK and Balb/c 3T3 cells. The plasmid pBORI lacks repeated sequences (14). The transfection was carried out in the presence of the seven expression plasmids encoding the HSV-1 replication genes (see “Materials and Methods”). DNA was isolated 48 h after transfection and subjected to DpnI and HindIII cleavage. Southern blots of agarose gels were probed using randomly primed pUC19 DNA. A, an autoradiograph of the Southern blots, B, the autoradiograph was analyzed using a PhosphorImager. The amount of replicated DNA obtained using circular p2aLORI in BHK cells was assigned a value of 100% and compared with the amount of replicated DNA from linearized plasmids in the same cells. The amount of replicated DNA for p2aLORI was assigned a value of 100% in Balb/c 3T3 cells and compared with the amount of replicated DNA from linearized plasmids in Balb/c 3T3 cells. The structures of the substrate molecules are presented schematically. To facilitate comparison, a star indicates the lanes in which the replication of linear p2aLORI was analyzed as well as the columns that show the relative amount of replicated p2aLORI DNA determined by PhosphorImager analysis.

**FIG. 8.** The position of directly repeated sequences in linear DNA determine the efficiency of homologous recombination in 3T3 cells. Circular and linear DNA containing directly repeated sequences either with a λ DNA spacer, p2aLORI, or without a λ spacer, p2aORI and p2bORI, were transfected into BHK and Balb/c 3T3 cells. The plasmid pBORI lacks repeated sequences (14). The transfection was carried out in the presence of the seven expression plasmids encoding the HSV-1 replication genes (see “Materials and Methods”). DNA was isolated 48 h after transfection and subjected to DpnI and HindIII cleavage. Southern blots of agarose gels were probed using randomly primed pUC19 DNA. A, an autoradiograph of the Southern blots, B, the autoradiograph was analyzed using a PhosphorImager. The amount of replicated DNA obtained using circular p2aLORI in BHK cells was assigned a value of 100% and compared with the amount of replicated DNA from linearized plasmids in Balb/c 3T3 cells. The structures of the substrate molecules are presented schematically. To facilitate comparison, a star indicates the lanes in which the replication of linear p2aLORI was analyzed as well as the columns that show the relative amount of replicated p2aLORI DNA determined by PhosphorImager analysis.

**DISCUSSION**

In this paper, we have examined the interdependence of DNA replication and recombination during the infectious cycle of Herpes simplex virus type I. We have also looked at cellular factors and conditions that affect homologous recombination. We find that homologous recombination occurs readily in BHK cells in the absence of viral DNA replication and virus-encoded gene products. Furthermore, the products of homologous recombination appear to be very efficiently utilized by replication and transcription machineries. In Balb/c 3T3 cells, linear DNA molecules with nonhomologous sequences at the ends do not recombine, and they induce p53-dependent apoptosis. On the other hand, linear DNA molecules with direct repeats at the ends of the molecule support homologous recombination, and they are replicated by the HSV-1 replisome in Balb/c 3T3 cells. Our discussion will focus on three aspects of this work: the mechanisms for a coupling between DNA replication and recombination in virus infected cells; the regulation of recombination by p53; and the role of homologous recombination during the infectious cycle of herpes viruses.

We have noted that in the absence of DNA synthesis only small amounts of recombination products are detected. However, the products of homologous recombination appear to be readily amplified by the HSV-1 replication apparatus and to be efficiently used by the cellular transcription machinery. It therefore appears as if a subset of the transcribed DNA molecules display a high probability of simultaneously undergoing homologous recombination, DNA replication, and transcription in BHK cells. In other words, our observations suggest that these processes are to some extent coupled. Different models can account for the postulated coupling between homologous DNA sequences in p2aLORI resulted specifically in a 10-fold reduction in the efficiency of circularization and replication in Balb/c 3T3 cells (Fig. 8A). Our results suggest that Balb/c 3T3 cells transfected with p2aORI and p2bORI may have escaped apoptosis. Two interpretations are possible. Homologous recombination may still take place in Balb/c 3T3 cells, but the pathway that permits recombination repair of linear DNA containing direct repeats at an internal position is suppressed in these cells. The alternative interpretation would suggest that unique DNA ends induce apoptosis much more rapidly than DNA ends containing direct repeats.

**TABLE I**

| Cells          | Apoptotic cells* |
|----------------|------------------|
|                | Circular plasmid | Linear plasmid |
| 3T3            | 9.7             | 41              |
| 3T3.SV-T2      | 6.3             | 29              |
| MEF            | 8.9             | 38.8            |
| MEF p53−/−     | 8.6             | 12.5            |
| BHK            | 19              | 17              |
|                | 16.3            | 15.5            |

* Cells were transfected with 0.8 μg of circular or linear pCMVβ plasmid DNA encoding β-galactosidase. Apoptosis was measured after staining with 5-bromo-4-chloro-3-indolyl-β-D-galactoside. The ratio of apoptotic shrunken cells with pyknotic nuclei expressing β-galactosidase to the total number of cells expressing β-galactosidase was calculated for each cell line.

**TABLE II**

| Cells          | Transfected cells* |
|----------------|--------------------|
|                | Circular plasmid   | Linear plasmid   |
| 3T3            | 40                 | <1               |
| 3T3.SV-T2      | 30                 | 4                |
| MEF            | 30                 | 5                |
| MEF p53−/−     | 50                 | 40               |
| BHK            | 40                 | 10               |
|                | 40                 | 20               |

* Cells transfected with 0.8 μg of circular or linear pCMVβ plasmid DNA. Cells expressing β-galactosidase were identified by staining with 5-bromo-4-chloro-3-indolyl-β-D-galactoside.
recombination and DNA synthesis. In prokaryotic organisms homologous recombination may support the formation of productive replication forks (25). However, we have not been able to demonstrate a similar phenomenon in HSV-1 infected cells. DNA replication remained origin-dependent under all of our experimental conditions, and homologous recombination did not appear to stimulate DNA synthesis. A more likely explanation of the coupling between DNA replication and homologous recombination would be that these processes are spatially and temporally coordinated. In its simplest form, we might assume that most DNA molecules recombine and replicate as soon as they reach the nucleus. Another hypothesis would state that the interactions between different pathways of DNA metabolism depend on the nonrandom distribution of replication and recombination proteins into nuclear foci (35–39). Still another possibility would be that viral DNA replication and recombination are coordinated events in the cell cycle.

It is a striking fact that homologous recombination is easily demonstrated in BHK cells using functional assays. This is unexpected, since nonhomologous end joining is considered to be a more frequent process for double strand break repair in mammalian cells (9, 20). More recently, however, it has been demonstrated that homology-directed repair constitutes a major repair pathway in mammalian cells (18, 40). In this paper, we demonstrate that homologous recombination is regulated differently in BHK cells and Balb/c 3T3 cells. For example, DNA molecules with ends that cannot directly take part in homologous recombination failed to recombine in Balb/c 3T3 cells, and they were shown to induce apoptosis. In contrast, recombination and replication of linear DNA with the direct repeats located at the very ends of the molecules were readily detected in Balb/c 3T3 cells. In BHK cells, on the other hand, homologous recombination did not depend on the location of the repeated sequences.

We also demonstrated that homologous recombination in Balb/c 3T3 cells was controlled by p53. However, p53 cannot be the only factor governing the outcome of recombination reactions. BHK cells are immortalized cells that appear to express wild type p53 (34). T antigen from BK virus binds to p53 in BHK cells and greatly increases the half-life of the protein (41). BHK cells transformed with T antigen can now form foci in soft agar (41). Since BHK cells and Balb/c 3T3 cells react differently to our recombination substrates, other factors must contribute to the regulation of homologous recombination. A separate indication that other factors must act together with p53 to regulate recombination comes from studies on gene amplification. Drug-resistant colonies containing amplified genes are easily selected from BHK cells, but DNA amplification leading to drug-resistance is rare in normal human cells (42, 43).

A functional coupling between homologous recombination and p53 has also been observed in other instances (16, 44, 45). What are the mechanisms connecting p53 to homologous recombination? It is tempting to speculate that a direct communication between the apparatus responsible for homologous recombination and p53 exists. The protein-protein interaction between Rad51 and p53 has also been observed in other instances (16, 44, 45). Alternatively, the ability to distinguish between recombination substrates and relay that information to p53 and other components of the cell cycle machinery might rely on the use of signaling proteins such as DNA-dependent protein kinase and ATM-related kinases (26, 27, 29–31). A conceivable consequence might be that the induction of apoptosis could be suppressed by successful initiation of homologous recombination.

There are several steps in the life cycle of Herpes simplex virus type I and other herpes viruses that may involve cellular recombination pathways. The viral genomes are linear molecules. They have in many instances directly repeated sequences at their termini. The viral genomes have to be circularized to support a productive infection (6). Homologous recombination may be directly responsible for the circularization of viral genomes. Homologous recombination involving the repeated sequences at the ends of virus chromosomes may, in fact, be one way of evading an apoptotic response by the cell. Our observation that only linear molecules that present repeated sequences at the very ends of the DNA molecule are recombined and replicated in Balb/c 3T3 cells supports this notion. It is important to remember, however, that circular DNA molecules may also be formed by direct ligation of complementary DNA ends. An in vitro system that accurately performs direct ligation has recently been described (10). DNA ligase IV/Xrc4, Ku70, Ku86, and the catalytic subunit of DNA-dependent protein kinase are essential components of this system. In the event that the site of fusion would be altered, the virus still has a possibility to select correct products in the cleavage and packaging stages. There is also the possibility that homologous recombination coupled to gene conversion may edit the products of the ligation event. Cell lines that lack recombination proteins are now becoming available. It should therefore be possible to establish genetically the pathway that leads to circularization of herpes viruses.

A separate function of homologous recombination is to repair strand breaks occurring during the replication of HSV-1. As a result, a high frequency of recombination between genetic markers on different HSV-1 strains would be expected from coinfection experiments. Abundant experimental evidence demonstrates this phenomenon (7, 47, 48). Interestingly, however, homologous recombination between cellular chromosomes in mitotically growing mammalian cells is very rare (49). Possibly, chromatin structure will suppress homologous recombination in higher cells. Studies of recombination taking place during viral replication cycles might be a relatively accessible way of studying mechanisms of homologous recombination in mammalian cells and identifying the genes and gene products that control recombination.

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