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Roles of host cell factors in circularization of retroviral DNA☆

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Abstract

Early during retroviral infection, a fraction of the linear reverse-transcribed viral DNA genomes become circularized by cellular enzymes, thereby inactivating the genomes for further replication. Prominent circular DNA forms include 2-long-terminal repeat (LTR) circles, made by DNA end joining, and 1-LTR circles, produced in part by homologous recombination. These reactions provide a convenient paradigm for analyzing the cellular machinery involved in DNA end joining in vertebrate cells. In previous studies, we found that inactivating components of the nonhomologous DNA end-joining (NHEJ) pathway—specifically Ku, ligase 4, or XRCC4—blocked formation of 2-LTR circles. Here we report that inactivating another NHEJ component, the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), had at most modest effects on 2-LTR circle formation, providing informative parallels with other end-joining reactions. We also analyzed cells mutant in components of the RAD50/MRE11/NBS1 nuclease and found a decrease in the relative amount of 1-LTR circles, opposite to the effects of NHEJ mutants. In *MRE11*-mutant cells, a *MRE11* gene mutant in the nuclease catalytic site failed to restore 1-LTR circle formation, supporting a model for the role of MRE11 in 1-LTR circle formation. None of the cellular mutations showed a strong effect on normal integration, consistent with the idea that the cellular pathways leading to circularization are not involved in productive integration.
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Introduction

Several host cell DNA repair pathways influence the fate of retroviral DNA (for reviews see Bushman, 2001; Coffin et al., 1997). During normal integration, the linear DNA product of reverse transcription first becomes connected to host cell DNA on one strand only at each end by the virus-encoded integrase enzyme, forming an integration intermediate (Fig. 1). Unpairing of the host cell DNA between the points of joining results in gaps at each host-virus DNA junction and a short unpaired 5′ flap. DNA repair is required to fill in the gap, trim off the 5′ overhang, and ligate the unjoined strand, forming the integrated provirus. This reaction can be modeled in vitro with known cellular gap repair enzymes (Yoder and Bushman, 2000), though the factors active during infection in vivo have not been fully clarified.

Linear viral DNA molecules can also become circularized by at least four pathways (Brown, 1997; Shoemaker et al., 1980), reactions that prevent subsequent integration of the viral DNA. In the first, the unintegrated linear viral DNA becomes circularized by ligation of the two ends, forming a 2-long-terminal repeat (LTR) circle. The cellular nonhomologous DNA end-joining (NHEJ) factors Ku80, XRCC4, and ligase 4 have been shown to be required for this process (Jeanson et al., 2002; Li et al., 2001). In the second pathway, homologous recombination between the LTRs results in formation of a 1-LTR circle (Farnet and Haseltine, 1991). Host cell factors important in this pathway have not been defined. In the third pathway, 1-LTR circles can also be formed from reverse transcription intermediates that stall before completing synthesis of the linear DNA (see Miller et al., 1995 for details and references). In the fourth pathway, the viral DNA can use itself as an integration

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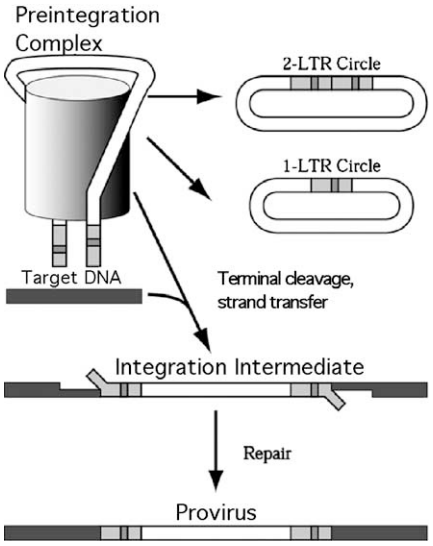

Fig. 1. Transformations of retroviral DNA early during infection. The linear product of reverse transcription can become integrated, to form the provirus, or circularized to form 1-LTR or 2-LTR circles. See text for details.

target, yielding rearranged circular forms (Shoemaker et al., 1980). In addition to these fates, much of the viral DNA can also be degraded after synthesis (at least in the high-titer infections of cultured cells that are often studied) (Bell et al., 2001; Butler et al., 2001; Zennou et al., 2000). Several further proposals have also been made for DNA repair factors acting on retroviral DNA (Brin et al., 2000; Mulder et al., 2002).

Here we report studies of HIV DNA circularization in cells mutant in genes encoding the DNA repair proteins DNA-dependent protein kinase catalytic subunit (DNA-PKcs), Mre11, and NBS1. DNA-PKcs is a component of the cellular NHEJ system that participates in some but not all NHEJ reactions (Bassing et al., 2002; Fugmann et al., 2000; Lieber, 1998). The Rad50/Mre11/NBS1 complex is implicated in DNA repair by homologous recombination, checkpoint control, telomere maintenance, and meiosis (D'Amours and Jackson, 2002; Tauchi et al., 2002). We have compared the relative amounts of 1-LTR and 2-LTR circles recovered after infection of mutant and normal control cells, allowing potential roles for these cellular DNA repair factors to be inferred. We have also assessed the effects of these cellular mutants on titers of infecting retroviral vectors and on the toxicity to cells of those infections.

Results

Circularization of retroviral genomes in cells mutant in DNA-PKcs

The NHEJ protein DNA-PKcs binds Ku70 and Ku80, thereby aiding in apposition of DNA ends at double-strand breaks. This complex then recruits ligase 4 and its cofactor XRCC4 to ligate the broken DNA ends. DNA-PKcs is also implicated in the signaling that coordinates the repair of DNA damage (Lieber, 1998). The role of DNA-PKcs in retroviral integration has been controversial (Baekelandt et al., 2000; Coffin and Rosenberg, 1999; Daniel et al., 1999, 2001a, 2001b; Taganov et al., 2001). Here we report that DNA-PKcs has at most a limited effect on 2-LTR circle formation, in contrast to Ku80, XRCC4, and ligase 4, which are required (Li et al., 2001).

To investigate whether DNA-PKcs is involved in circularization of unintegrated retroviral DNA, cells mutant in the *DNA-PKcs* gene were infected with an HIV-based vector and circularization was assessed. The HIV-based vector particles used were made by transfection of three plasmids into 293T cells (Naldini et al., 1996). One encoded HIV *gag-pol*, the second encoded VSV-G envelope, and the third encoded a packageable genome encoding enhanced green fluorescent protein (*eGFP*) (Fig. 2A). The forms of unintegrated DNA present were then assessed by isolation of episomal DNA from infected cells, cleavage with restriction enzymes, separation of DNA fragments by electrophoresis, and analysis by Southern blotting using a probe recognizing the HIV LTR sequence. Cleavage with the restriction enzymes used yielded diagnostic DNA fragment lengths for the 2-LTR circle, 1-LTR circle, and left and right ends of the linear genome.

Three *DNA-PKcs* mutant cell lines were analyzed along with matched controls. MO59K and MO59J are human cells derived from the same tumor, with the latter line mutant for *DNA-PKcs*, while the former has functional *DNA-PKcs*. 2-2 and SF19 are murine cells, in which the latter is mutant in the *DNA-PKcs* gene and the former is complemented with a human copy of the gene (Kienker et al., 2000). The 1821 cells are a fibroblast line derived from Arabian horses that are immunodeficient due to *DNA-PKcs* mutations; these are compared to wild-type horse fibroblasts (Wiler et al., 1995). As a control, a cell line deleted for the gene encoding DNA ligase 4 (Nalm-6 $LIG4^{-/-}$) and its matched wild-type control (Nalm-6) were also tested, since the mutation in Nalm-6 $LIG4^{-/-}$ has been reported previously to block circularization of retroviral DNA (Li et al., 2001).

The Nalm-6 $LIG4^{-/-}$ cells did not support efficient formation of 2-LTR circles, as reported previously (Fig. 2B, lanes 1 and 2). In contrast, all three *DNA-PKcs* mutant lines did support detectable formation of 2-LTR circles (Fig. 2B, lanes 3–8). The ratio of 2-LTR circles to 1-LTR circles is shown below the autoradiogram for each lane. Wild-type cell lines showed ratios ranging from 0.16 to 0.43 2-LTR circles per 1-LTR circle (though we note that the ratios for each individual cell line were quite consistent). The quantitation presented represents the raw values measured minus background. Note, however, that the two LTR circular molecules will bind twice as many of the labeled LTR probe sequences, and so the signal is doubled for this DNA form. The mutation in the Nalm-6 $LIG4^{-/-}$ line reduced 2-LTR

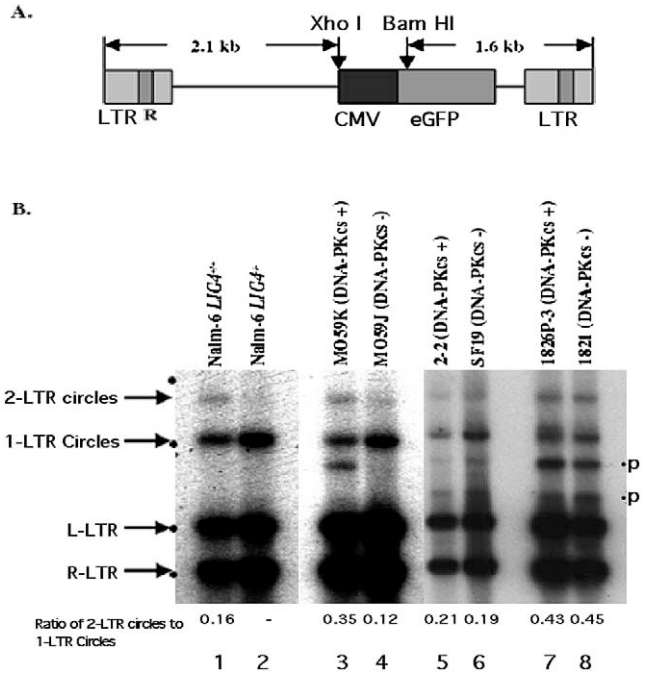

Fig. 2. Viral DNA circularization in cells mutant in ligase 4 or DNA-PK-mutant and matched wild-type controls. (A) Structure of the SkSM2 HIV-based vector (Hansen et al., 1999) used in these experiments. The *Xho*I and *Bam*HI sites used to cleave the DNA for analysis by Southern blot are indicated. (B) Southern blot analysis of extrachromosomal DNA isolated from cells infected with the HIV-based vector. The mobilities of DNA fragments derived from 1-LTR circles, 2-LTR circles, the left end of the linear viral DNA (L-LTR), and the right end of the viral DNA (R-LTR) are indicated. Cell types are indicated above the autoradiograms. The ratios of 2-LTR circles to 1-LTR circles are indicated. Size markers are indicated to the left of the autoradiogram; sizes were (from the top, in kb) 4, 3, 2, and 1.6. "P" (partial digestion products) indicates DNA fragments with sizes matching those expected for products of incomplete restriction enzyme cleavage of the viral DNA. The dash in lane 2 indicates that 2-LTR circles were undetectable and so the ratio could not be quantitated. Infections were carried out at high titer (>5 infectious units per cell as measured by titration of vector stocks on 293T cells).

circle formation to below the level of detection. Comparison of the MO59K and MO59J cells revealed that circularization was reduced about threefold (Fig. 2B, compare lanes 3 and 4). For the other two lines there was no significant reduction in 2-LTR circles in the mutant lines (Fig. 2B, compare lanes 5 to 6 and 7 to 8). These data indicate that DNA-PKcs is not strictly required for formation of 2-LTR circles in these cells, and in two of three cell lines the mutation in *DNA-PKcs* had no detectable influence. The third line, MO59J, is known to express low levels of *ATM*, which may possibly contribute to the phenotype. We return to the mechanistic implications of these findings under the Discussion.

Circularization of retroviral genomes in cells mutant in MRE11 and NBS1

In an effort to identify further factors important for circularization of retroviral DNA, we analyzed infections of cells mutant in two components of the Rad50/Mre11/NBS1 complex. The first cell line studied, ATLD3, was derived from human patients with ataxia telangectasia-like disease (ATLD), which results from diminished activity of Mre11. As a control, the cell line was complemented with a cloned copy of the *MRE11* gene. Previous work has shown that the gene is expressed and complements a phenotype of the *MRE11* defect (Stracker et al., 2002). The analysis of this cell line was complicated by the sluggish growth of the mutant line, requiring very high titer infections (multiplicity of infection 50 particles per cell) to obtain enough material to analyze. In the complemented cell line, the 2-LTR circle forms are barely detectable, while 1-LTR circles are abundant (Fig. 3, lane 1). In the *Mre11* mutant line, in contrast, 2-LTR circles are increased in abundance and 1-LTR circles are decreased (Fig. 3, lane 2). Thus the defect in Mre11 has the opposite effect of defects in the "core" NHEJ proteins (ligase 4, XRCC4, and Ku), favoring formation of 2-LTR circles at the expense of 1-LTR circles.

We note that the ATLD3/MRE11-complemented cell line showed unusually low levels of 2-LTR circles compared to other lines we have studied. We cannot exclude the possibility that abnormally high levels of Mre11 activity are present in the complemented cells, and that this resulted in diminished formation of 2-LTR circles and increased formation of 1-LTR circles. If so, this would still be an indication that the Rad50/Mre11/NBS1 complex promotes formation of 1-LTR circles.

Circularization was also assayed in an ATLD3 cell line containing a modified MRE11 gene that was mutant in the nuclease active site (Fig. 3, lanes 3 and 4). No effect on viral DNA circularization was seen upon introduction of the

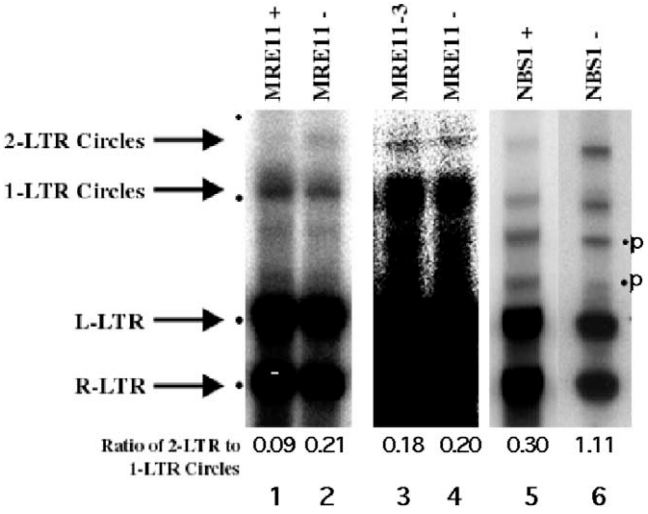

Fig. 3. Circularization of retroviral DNA in cells defective in Rad50/MRE11/NBS1. MRE11+ and − indicate the ATLD3 cell line (−) or a subline complemented with the wild-type human MRE11 gene (+). MRE11-3 is an ATLD3 derivative containing an MRE11 gene mutant in the region encoding the nuclease active site. IMR90 is a normal human fibroblast line (NBS1+). NBS1− indicates fibroblasts from an *NBS*-mutant patient. Other markings are the same as in Fig. 2.

Table 1
Titers of retroviral vectors on cells mutant in genes encoding DNA repair proteins

Gene	Mutant cell line (Control cell line)	Titer of mutant as a % of the control (SD)[Infection method]	Range of m.o.i. studied	Selective toxicity of infection in mutant?	Organism
Ku80	xrs6 (xrs6+huKu80)	73% (25%)[1,2]	1.6–80	Sporadic at highest titer	Hamster
	XRV 15B (V79)	137%[1]	0.1	No	Hamster
DNA-Pkcs	MO59J (MO59K)	144% (56)%[2,3]	0.4–10	No	Human
	S33 (N2)	296% (190%)[3]	1–25	No	Mouse
	SF19 (SF19+huDNA-PKcs)	183% (57%)[2,3,4]	2–10	No	Mouse
	1821 (1826P-3)	152% (43%)[2,3]	0.4–15	No	Horse
Ligase 4	Nalm-6 LIG4$^{-/-}$ (Nalm-6 LIG4$^{+/+}$)	90% (32%)[2,3]	1–10	Yes (increases with titer)	Human
MRE11	ATLD3 (ATLD3+MRE11)	96% (21%)[5]	1–25	No	Human
	ATLD3+MRE11-3 (ATLD3+MRE11)	139% (30%)[5]	1–25	No	Human
NBSI	NBS (IMR90)	57% (18%)[3]	1–25	No	Human

Note. The numbered protocols for virus titration are described under infection methods in the Materials and methods.

mutant *MRE11* gene, though the protein was properly expressed (Stracker et al., 2002). This finding supports the hypothesis that the nuclease activity is important for formation of 1-LTR circles.

Cells were also tested from a patient with Nijmegen breakage syndrome, which are hypomorphic for the NBS protein. Normal human fibroblasts (IMR90) were assayed in parallel for comparison. Infections were carried out with the HIV-based vector and extrachromosomal forms assayed by Southern blotting as above (Fig. 3, lanes 5 and 6). The ratio of 2-LTR circles to 1-LTR circles in the *NBS1*-mutant cell line differed from any previously analyzed, displaying equal abundance of 1-LTR and 2-LTR circles. The ratio for the control IMR90 fibroblasts was 0.3 (2-LTR circle/1-LTR circles), in the typical range for the wild-type cell lines studied. Thus the *NBS1* mutation increased the ratio of 2-LTR to 1-LTR circles. In summary, for both cell lines tested, mutations in the Rad50/Mre11/NBS1 complex altered the ratio of circular forms in favor of 2-LTR circles.

Efficiency of retroviral infection in cells impaired in DNA repair

Aspects of the role of the NHEJ system in retroviral replication have been controversial. Daniel and co-workers proposed that the NHEJ system plays a role in the gap repair step late after retroviral infection (Baekelandt et al., 2000; Coffin and Rosenberg, 1999; Daniel et al., 1999, 2001a, 2001b; Taganov et al., 2001). This inference was supported by the proposal that retroviral infection of cells mutant in NHEJ components led to apoptosis of mutant cells. A retrovirus mutant in integrase did not cause the apparent apoptotic response, leading these workers to propose that the NHEJ system was involved in repair of DNA gaps created by the first steps of integration (Fig. 1). However, two other groups did not reproduce the reported selective killing in DNA-PKcs mutant cells (Baekelandt et al., 2000 and data cited in Coffin and Rosenberg, 1999). In another study, human lymphoid cells mutant in ligase 4 were found to display a consistent increase in apoptosis after infection in the mutant cells (Li et al., 2001). In this case integration was not required for toxicity, though reverse transcription was. This led to the conclusion that the unintegrated DNA generated a cytopathic signal leading to apoptosis in this model, consistent with previous proposals (Temin et al., 1980).

Table 1 summarizes the outcome of 78 separate infections of cells mutant in genes encoding NHEJ or Rad50/Mre11/NBS1 proteins. Cells were infected with either of two HIV-based vector transducing the gene for *eGFP* or an MLV-based vector transducing genes for puromycin resistance and β-galactosidase. The only reproducible selective killing of mutant cells seen was with the Nalm-6 *LIG4*$^{-/-}$ line in the presence of high titers of virus. No other mutant line displayed reproducible selective cytotoxicity after infection, though sporadic cytotoxicity was seen in the case of Ku-mutant cells. For the NBS/IMR90 pair, cytotoxicity was seen for both mutant and wild-type at very high levels of infection (data not shown).

Another way of asking whether cellular DNA repair factors are important for normal infection is by comparing the titers of retroviral stocks on mutant and wild-type cells. The titers of retroviruses were also not substantially different in the paired mutant and wild-type lines studied (Table 1). The NBS cells showed slightly lower titers than the IMR90 normal fibroblast control (about twofold), but we note that the NBS cells grew quite slowly, while the IMR90 cells showed more robust growth, making the modest difference detected difficult to interpret. Even in the case of high-titer infections of ligase 4-mutant cells, in which cell killing was seen, the retroviral titer did not differ on the surviving cells. Killing due to integration would predict that cells undergoing integration should be selectively lost, resulting in reduced titer, but this was not seen. Thus this observation provides independent evidence against the view that cytotoxicity in the ligase 4-mutant results from the integration process.

To strengthen this point further, the abundance of integrated proviruses in ligase 4-mutant or wild-type Nalm-6 cells was analyzed by Southern blotting. Cells were passaged for 3 weeks after infection, which eliminates any

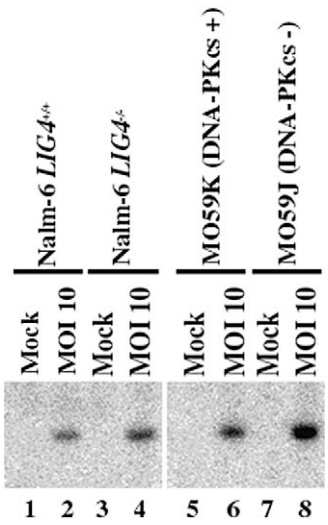

Fig. 4. Equivalent accumulation of integration products in mutant and wild-type cells defective in ligase 4 or DNA-PK-cs. The cell types indicated above the autoradiograms were infected with the HIV-based vector described in the Table 1 legend (infection method 1) at an m.o.i. of 10 (determined by titration on 293T cells); then cells were grown for 3 weeks to eliminate any unintegrated viral DNA. Genomic DNA was then isolated with *Bam*HI and *Eco*RI to liberate an internal fragment of the viral genome (10 μg per lane) and were then analyzed by Southern blotting using a probe that recognized the internal *Bam*HI to *Eco*RI viral DNA fragment.

unintegrated forms of the viral DNA by dilution during cell growth (Butler et al., 2001). Cellular DNA was then isolated, cleaved with restriction enzymes that liberate an internal fragment of the viral DNA, and analyzed by Southern blotting (Fig. 4). This revealed that there was no difference in the abundance of proviruses by this assay, paralleling the studies using *GFP* transduction by the HIV-based vector. This observation thus argues against the view that toxicity in the Nalm-6 *LIG4*$^{-/-}$ cells arises from integration. It is known that many more cells usually receive viral DNA than ultimately complete integration (Bell et al., 2001; Butler et al., 2001; Zennou et al., 2000), so the unintegrated DNA is a more satisfactory potential source of the proapoptotic signal in the ligase 4-mutant cells. Similar results were obtained for the MO59K and MO59J cell lines. In summary, these data argue against the idea that the NHEJ and Rad50/Mre11/NBS1 pathways are required for productive integration of retroviral DNA.

Discussion

We have investigated the effects of three DNA repair proteins, DNA-PKcs, Mre11, and NBS1, on circularization of retroviral DNA. Mutations in the core NHEJ factors Ku80, ligase 4, and XRCC4 were previously reported to drastically reduce formation of 2-LTR circles (Li et al., 2001). In contrast, we report here that mutations in the gene for DNA-PKcs, another NHEJ protein, do not strongly affect 2-LTR circle formation. We also report that mutations in MRE11 or NBS1 have an opposite effect compared to mutations in the core NHEJ factors, altering the ratio of circular forms in favor of 2-LTR circles. None of these cellular mutations strongly affected the titers of retroviral vectors, indicating that the encoded proteins are not strictly required in the productive integration pathway.

Why is the DNA-PKcs dispensable for the DNA end joining reaction that forms 2-LTR circles? A potentially informative parallel is provided by studies of V(D)J recombination, the site-specific recombination process that assembles antigen-receptor genes from their component gene segments in developing lymphocytes (Bassing et al., 2002; Fugmann et al., 2000; Lieber, 1998). During VDJ recombination, two DNA breaks are introduced, generating four DNA ends with distinct structures. The two coding ends have covalently sealed termini (hairpins), whereas the two signal ends have blunt and 5′-phosphorylated termini. Rejoining of the cleaved DNA ends is carried out by the cellular NHEJ system. Joining of coding ends requires DNA-PKcs, but joining of signal ends can proceed in the absence of DNA-PKcs (though there is considerable variation in the efficiency of signal end joining in different *DNA-PKcs* mutant animals and cell lines) (Meek et al., 2001; Shin et al., 1997; Wiler et al., 1995). DNA-PKcs has been suggested to be required at coding ends to bind and recruit the Artemis protein, a nuclease that opens DNA hairpins and trims off long DNA overhangs. These steps are required to allow subsequent ligation, a point made by convincing biochemical experiments (Ma et al., 2002). Retroviral DNA ends do not contain hairpins or long single-stranded regions (Fujiwara and Mizuuchi, 1988; Miller et al., 1997; Roth et al., 1989) and so resemble V(D)J signal ends but not coding ends. Thus the simple covalent structure at the viral DNA ends may explain the lack of requirement for DNA-PKcs, since there is apparently no need for processing by the Artemis nuclease.

How do the mutations in Rad50/Mre11/NBS1 alter the ratio of 1-LTR to 2-LTR circles? A simple model to explain these findings posits that the Rad50/Mre11/NBS1 complex is mechanistically involved in formation of 1-LTR circles, and in the absence of the complex, genomes are channeled into the 2-LTR circle pathway. An MRE11 gene mutant in the nuclease active site failed to complement the ATLD3 mutant cells for 1-LTR circle formation. This is consistent with a model in which the Mre11 nuclease promotes formation of 1-LTR circles by catalyzing degradation of one DNA strand at each end of the linear viral DNA, allowing annealing of the resulting single-stranded LTR sequences at each end. Repair of the resulting joint molecule would yield a 1-LTR circle. In support of this idea, a recent study of DT40 cells mutant in Nbs1 concluded that Nbs1 is important for homologous recombination in vertebrates (Tauchi et al., 2002). Alternative models cannot be excluded, however. For example, the MRE11 complex might act by inhibiting

formation of 2-LTR circles, thereby promoting formation of 1-LTR circles indirectly.

The *MRE11* mutation does not entirely eliminate 1-LTR circle formation. This could be because the mutant is hypomorphic, and residual activity may suffice for some 1-LTR circle formation. Further pathways also likely contribute—1-LTR circles are known to be formed from stalled reverse transcription intermediates (see Miller et al., 1995 and references therein), potentially accounting for the remaining 1-LTR circles produced in the mutant cells.

Assays of retroviral DNA circularization provide a relatively simple assay for monitoring DNA end-joining reactions. As an example of the utility of such assays, we have recently used retroviral infection to study end joining in chicken DT40 cells, which are highly active for homologous recombination and so popular for use in constructing gene knockouts (Winding and Berchtold, 2001). We found that retroviral infections of DT40 cells yielded very few 2-LTR circles but abundant 1-LTR circles (data not shown). This supports a model in which DT40 cells achieve high rates of homologous recombination by suppressing NHEJ, thereby allowing homologous recombination to compete more effectively, a conclusion also supported by experiments of others (Fukushima et al., 2001).

Materials and methods

Infection methods

Infection method 1

The HIV-based vector particles used were produced from the cell line SkSM2 as described (Hansen et al., 1999; Kafri et al., 1999). The cell line supplies HIV *gag-pol* and the VSV-G envelope under Tet-inducible control and expresses constitutively an HIV-based vector component expressing *eGFP* under control of the CMV promoter. To carry out infections, 2×10^4 cells were plated in 24-well plates 16–20 h prior to transduction; 10–0.016 μl of the HIV-1-based vector particles (typically about 10^4 infectious units per nanogram p24 on 293T cells) transducing *gfp* was added to the cells in a five-fold dilution series. For infection of some cell types, DEAE dextran (5–10 μg/ml) or polybrene (4–8 μg/ml) was added. Infected centers were counted under a fluorescence microscope 48–72 h posttransduction.

Infection method 2

Infection method 2 differed from infection method 1 in that GFP-positive cells were scored by FACS instead of counting positive cells. Either of two different GFP-transducing HIV-based vectors were used: one was the SkSM2-derived particles described under method 1 above. The second was made by triple transfection of pdeltaR9 (Naldini et al., 1996), pVSV-G (L. Li, personal communication), and p156RRLsinPPTCMVGFPPRE (Follenzi et al., 2000) into 293T cells. The latter plasmid encodes a packagable genome with deletions in the LTR (self-inactivating vector), a central polypurine tract, and the gene for *eGFP* under control of the CMV promoter. Cells were incubated with different concentrations of VSV-G-pseudotyped HIV-1-based vector particles (typically about 10^5 infectious units per nanogram p24 on 293T cells) at 37°C for 24 h. *GFP* expression was analyzed 1–14 days posttransduction by FACS.

Infection method 3

Method 3 is identical to method 2 except that cells were spin inoculated at 1200 *g* for 1 h with different concentrations of VSV-G-pseudotyped HIV-1-based vector particles, which enhances transduction (O'Doherty et al., 2000).

Infection method 4

The MLV-based vector was prepared by lipofectamine transfection of the 293 helper cell line HEK with 10 μg of the plasmid, pBABE-puro/*lac*Z (generous gift of Michele Battle). After 48 h, viral supernatants were collected and used to infect 2×10^5 murine or equine fibroblasts that had been plated 12 h earlier in 10-cm dishes. In each experiment, several viral dilutions were tested. Cells were placed under puromycin selection 48 h later. After 7–10 days of drug selection, plates were fixed and stained with X-gal, and colony numbers were assessed.

Infection method 5

HIV vector particles transducing the *LUC* gene were generated by transfection of pdeltaR9, pVSV-G, and p156RRLsinPPTCMVLUCPRE into 293T cells. The resulting HIV-luc vector particles were titrated on cells (volumes of 12.5, 2.5, and 0.5 μl of a stock of 160 ng p24/ml per 5×10^3 cells) and incubated at 37°C for 2 days. Cells were harvested and counted and cell lysates were assayed for luciferase activity. The HIV-luciferase transducing vector (p156RRLsinPPTCMVLUCPRE) was constructed as follows. The Luc+ gene from pGL3 Basic (Promega Corp.) was amplified by PCR with primers JH1 and JH2, which add a *Bam*HI site at the 3′ and a *Sal*I site at the 3′ end of the Luc+ amplicon. This PCR product was digested with *Bam*HI and *Sal*I and cloned into *Bam*HI/*Sal*I digested p156RRLsinPPTCMVGFPPRE.

JH1: GGA TCC ATG GAA GAC GCC AAA AAC ATA
JH2: GTC GAC TTA CAC GGC GAT CTT TCC GC

Purification of virions

Virions were purified essentially as described (Li et al., 2001). Supernatants containing HIV-based vectors were centrifuged briefly at low speed to pellet cellular debris and then filtered through 0.45-μm filters. Viral particles were then pelleted by centrifugation at 23,000 *g* and resuspended

in 1/30 volume of fresh medium. Recovery of viral particles was quantified using the DuPont p24 ELISA.

Southern blot analysis

Unintegrated viral DNA preparations were cleaved with *Xho*I and *Bam*HI; Southern blots were probed with labeled DNA encoding the HIV LTR as described (Li et al., 2001). Quantitation was carried out by PhosphorImager with empirical determination of background levels in similarly sized volumes in each gel lane. In the gels, the bands labeled "P" have the sizes expected for partial digestion products of restriction enzyme cleavage of the viral DNA genomes.

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