useful in aborting latent KSHV infection and in preventing or treating KSHV-associated diseases.

Note added in proof: The Z6 cis-acting element has been further localized to three copies of the terminal repeat and up to ~600 nucleotides of unique sequence.

References and Notes

1. Y. Chang *et al.*, *Science* **266**, 1865 (1994); P. S. Moore *et al.*, *J. Virol.* **70**, 549 (1996); S.-J. Gao *et al.*, *N. Engl. J. Med.* **335**, 233 (1996); P. S. Moore and Y. Chang, *ibid.* **332**, 1181 (1995).
2. E. Cesarman *et al.*, *Blood* **86**, 2708 (1995).
3. M. B. Rettig *et al.*, *Science* **276**, 1851 (1997); J. Soulier *et al.*, *Blood* **86**, 1276 (1995); E. Cesarman, Y. Chang, P. S. Moore, J. W. Said, D. M. Knowles, *N. Engl. J. Med.* **332**, 1186 (1995).
4. L. L. Decker *et al.*, *J. Exp. Med.* **184**, 283 (1996).
5. J. Yates, N. Warren, D. Reisman, B. Sugden, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3806 (1984); J. L. Yates, N. Warren, B. Sugden, *Nature* **313**, 812 (1985).
6. D. R. Rawlins, G. Milman, S. D. Hayward, G. S. Hayward, *Cell* **42**, 859 (1985).
7. S.-H. Kung and P. G. Medveczky, *J. Virol.* **70**, 1738 (1996).
8. J. J. Russo *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 14862 (1996).
9. P. Kellam *et al.*, *J. Hum. Virol.* **1**, 19 (1997); L. Rainbow *et al.*, *J. Virol.* **71**, 5915 (1997); D. H. Kedes, M. Lagunoff, R. Renne, D. Ganem, *J. Clin. Invest.* **100**, 2606 (1997); F. Neipel, J. C. Albrecht, B. Fleckenstein, *J. Virol.* **71**, 4187 (1997).
10. J. C. Albrecht *et al.*, *J. Virol.* **66**, 5047 (1992); H. W. Virgin IV *et al.*, *ibid.* **71**, 5894 (1997).
11. S. J. Gao *et al.*, *Nature Med.* **2**, 925 (1996); D. H. Kedes *et al.*, *ibid.*, p. 918; D. Jones *et al.*, *N. Engl. J. Med.* **339**, 444 (1998); R. Renne *et al.*, *Nature Med.* **2**, 342 (1996).
12. L. Szekely *et al.*, *J. Gen. Virol.* **79**, 1445 (1998).
13. FISH was performed with TSA-Direct (NEN Life Science Products). Colcemid treatment and hypotonic swelling of cells was performed as described in (*19*). Cells were fixed in 4% paraformaldehyde at room temperature for 10 min and then fixed in 70% ethanol at 4°C for 10 min. Fixed cells were permeabilized with 0.5% Triton-X 100 and overlaid with DNA in situ hybridization solution (DAKO, Carpinteria, CA) containing 20 ng of a probe that was labeled with biotin and nick translated from pBSLANA. pBSLANA was constructed by subcloning the 4.7-kb KSHV genomic Sac I fragment flanking ORF 73 from the L54 library phage (*8*) into pBluescript (Stratagene). After denaturation of DNA at 93°C for 5 min, slides were incubated for 4 hours at 37°C, washed in 0.2× standard saline citrate for 30 min at 45°C, and blocked in TNB (0.1 M tris-HCl, pH 7.5, 0.15 M NaCl, 0.5% blocking reagent) at room temperature for 30 min. Slides were then incubated with streptavidin–horseradish peroxidase (1:100) for 30 min at 37°C and incubated with cyanine 3-tyramide (1:50) for 10 min at room temperature. After FISH, cells were incubated with BJAB cell extract–adsorbed KSHV immune serum (1:50) followed by secondary fluorescein isothiocyanate–conjugated antibody and counterstained with DAPI (1 μg/ml) in methanol for 15 min. For two-color fluorescence, DAPI was omitted.
14. Genomic ORF 73 was cloned downstream of the simian virus 40 (SV40) promoter in pSG5 (Stratagene) (termed pSG5LANA) or in pSG5FLAG [E. Hatzivassiliou, P. Cardot, V. I. Zannis, S. A. Mitsialis, *Biochemistry* **36**, 9221 (1997)] (termed pSG5 F-LANA). In pSG5 F-LANA, the four NH_2-terminal LANA amino acids are replaced with the FLAG epitope (DYKDDDDKV) (*22*). LANA stable cell lines were generated by electroporating pSG5LANA or pSG5 F-LANA and a plasmid encoding the hygromycin-resistance gene downstream of an SV40 promoter into BJAB cells [K. M. Kaye, K. M. Izumi, G. Mosialos, E. Kieff, *J. Virol.* **69**, 675 (1995); D. Liebowitz, J. Mannick, K. Takada, E. Kieff, *ibid.* **66**, 4612 (1992)]. After 48 hours, cells were seeded into microtiter plates, and hygromycin-resistant clones were selected. Hygromycin-resistant BJAB cells expressing pSG5LANA or pSG5 F-LANA were transfected with 25 μg of the Z6 or Z8 cosmid. Z6 contains KSHV sequence including the terminal repeats and the first ~33 kb of the BC-1 genome cloned into S-Cos1 (Stratagene), and Z8 contains nucleotides ~73,000 to 107,000 of BC-1 KSHV cloned into S-Cos1 (*8*). S-Cos1 encodes the neomycin-resistance gene downstream of an SV40 promoter and provides G418 resistance. After 48 hours, cells were placed under G418 selection. For subclone analysis, the Z6 cosmid was digested with Hind III and religated, resulting in the deletion of KSHV sequences after the first ~13 kb of the genome (Z6-13). The two largest remaining Z6 KSHV Hind III fragments of ~7 and ~11 kb were cloned into pREP9 (Invitrogen) after the deletion of the pREP9 sequences between Cla I and Kpn I (Z6-7 and Z6-11 respectively). pREP9 encodes the neomycin-resistance gene downstream of a thymidine kinase promoter and provides G418 resistance. The Z6 subclones were transfected into BJAB/F-LANA or BJAB cells and selected for G418 resistance as above.
15. M. E. Ballestas and K. M. Kaye, unpublished data.
16. Gardella gels were prepared as previously described [T. Gardella, P. Medveczky, T. Sairenji, C. Mulder, *J. Virol.* **50**, 248 (1984)].
17. A. Bochkarev *et al.*, *Cell* **84**, 791 (1996).
18. D. Reisman and B. Sugden, *Mol. Cell. Biol.* **6**, 3838 (1986).
19. L. Petti, C. Sample, E. Kieff, *Virology* **176**, 563 (1990).
20. E. Grogan *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 7650 (1983); B. M. Reedman and G. Klein, *Int. J. Cancer* **11**, 499 (1973).
21. C. W. Lehman and M. R. Botchan, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 4338 (1998); M. H. Skiadopoulos and A. A. McBride, *J. Virol.* **72**, 2079 (1998); A. Harris, B. D. Young, B. E. Griffin, *ibid.* **56**, 328 (1985).
22. Single-letter abbreviations for the amino acid residues are as follows: D, Asp; K, Lys; V, Val; and Y, Tyr.
23. R. Renne, M. Lagunoff, W. Zhong, D. Ganem, *J. Virol.* **70**, 8151 (1996).

24. We thank E. Kieff for helpful discussions and P. Marks for assistance with microscopy for Fig. 2. This work was supported by grant CA67380-04 (K.M.K.) from the National Cancer Institute.

3 November 1998; accepted 23 March 1999

A Role for DNA-PK in Retroviral DNA Integration

René Daniel, Richard A. Katz, Anna Marie Skalka*

Retroviral DNA integration is catalyzed by the viral protein integrase. Here, it is shown that DNA-dependent protein kinase (DNA-PK), a host cell protein, also participates in the reaction. DNA-PK-deficient murine *scid* cells infected with three different retroviruses showed a substantial reduction in retroviral DNA integration and died by apoptosis. *Scid* cell killing was not observed after infection with an integrase-defective virus, suggesting that abortive integration is the trigger for death in these DNA repair–deficient cells. These results suggest that the initial events in retroviral integration are detected as DNA damage by the host cell and that completion of the integration process requires the DNA-PK–mediated repair pathway.

Integration is an essential step in retroviral replication (*1*). Processing (nicking) of the viral DNA 3′ ends and joining of these ends to staggered phosphates in the host DNA are carried out by the viral integrase (IN) protein (*2*). The initial linkage between viral and host DNA is a gapped intermediate in which the viral DNA 5′ ends are unjoined. The processing and joining steps in the integration reaction have been reconstituted in vitro, with purified retroviral integrases and model viral and host DNA substrates. Repair of the gaps in vivo results in a 4– to 6–base pair repeat of host DNA flanking each proviral end, but this final step has not yet been reproduced in vitro. It has been reported that inhibition of a host DNA repair–related protein, poly(adenosine diphosphate–ribose) polymerase, blocks retroviral integration (*3*). Although it is generally assumed that host cell repair enzymes complete the integration reaction, the pathways responsible and the mechanism by which repair is accomplished have not been identified.

In mammalian cells, repair of double-stranded DNA breaks by nonhomologous end joining (NHEJ) is mediated by DNA-dependent protein kinase (DNA-PK). DNA-PK is composed of a DNA-binding Ku70/Ku86 heterodimer and a large catalytic subunit, $DNA\text{-}PK_{cs}$ (*4*). DNA-PK also functions in V(D)J recombination, and the underlying genetic defect in the V(D)J recombination– deficient, severe combined immunodeficiency (*scid*) mouse (*5*) is a truncation mutation of $DNA\text{-}PK_{cs}$ (*6*). Thus, *scid* cell lines and primary cells are deficient in DNA-PK activity (*7*).

To investigate whether DNA-PK has a role in the repair process that completes retroviral integration, we infected *scid* cells with retrovirus vectors. *scid* pre-B cell lines S7, S29, and S33 and a control, normal cell line, N2 (*8*), were first infected with an avian retrovirus vector, encoding an amphotropic envelope protein that allows infection of a wide variety of mammalian cells [RCASBP-M(4070A), hereafter abbreviated R/M] (*9*). The results (Fig. 1A) were striking;

Institute for Cancer Research, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111, USA.

*To whom correspondence should be addressed. E-mail: AM_Skalka@fccc.edu

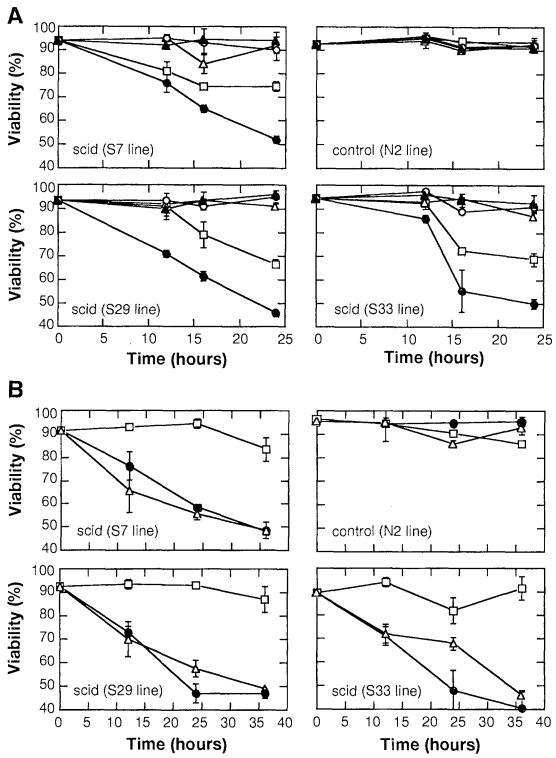
after infection with the vector at a multiplicity of ~2 infectious units (i.u.) per cell, viability (*10*) of the *scid* cells dropped to 40 to 50%, whereas the viability of the N2 cells remained >90%. Cell death was dependent on the virus concentration, and it was first observed 12 hours after infection, indicating that an early event in the retroviral replication cycle was responsible.

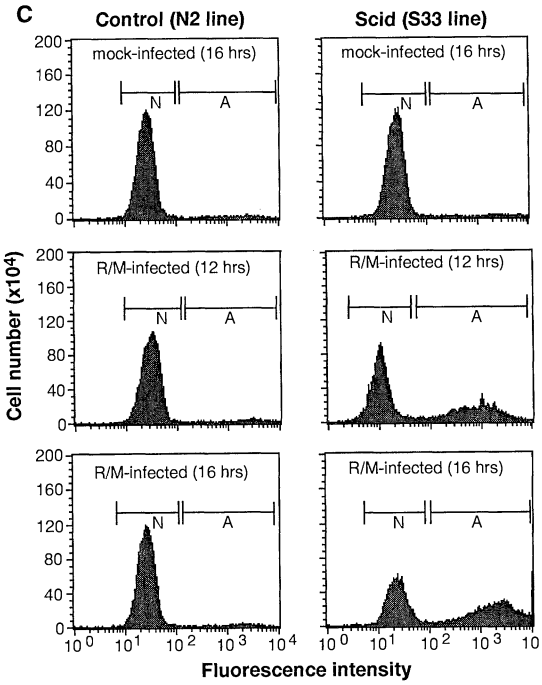
To investigate this phenomenon further, we constructed matched derivatives of the original avian retrovirus vector (*11*). One virus was integration-competent (IN^+); the other was integration-defective (IN^-), as it encoded a $Asp^{64} \rightarrow Glu$ (D64E) substitution in the conserved D,D35,E motif, which makes up the catalytic center of avian sarcoma virus (ASV) integrase. All other early steps, including synthesis of viral DNA and its entry into the nucleus, were normal in the IN^- mutant (*12*). Death of the *scid* cells occurred after infection with the IN^+ virus, but not the IN^- virus (Fig. 1B), suggesting that this response was dependent on retroviral IN activity and was not merely an antiviral response induced by uptake of virus.

scid cells are highly sensitive to DNA damage, which appears to trigger apoptosis (*13*). Retroviral infection seemed to elicit a similar response. Application of the TdT-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay confirmed that infection of *scid* cells with the avian virus vector induced apoptosis in about 33 to 45% of infected cells at 12 and 16 hours after infection, respectively (Fig. 1C) (*14*). In contrast, no increase in apoptosis was detected after infection of the N2 cells. Similar results were obtained when apoptosis was evaluated by detection of cytoplasmic histone-associated DNA fragments and increase in caspase-3 activity, a marker for early events in apoptosis (*12, 15*). Thus, we conclude that the retrovirus-infected *scid* cells die by apoptosis.

We considered the possibility that the *scid* cell lines may have distinct phenotypes due to their lymphoblastoid lineage, immortalization, or passage history. Therefore, the efficiency of stable retrovirus integration was tested in *scid* mouse embryo fibroblasts (*16*) with the IN^+ vector that carries a neomycin resistance gene (neo^r) (*11*). The number of stable G418-resistant colonies was 10-fold lower in the infected *scid* fibroblasts than in matched normal primary mouse embryo fibroblasts (Table 1). We also tested the ability of the same vector to integrate in cell lines that are deficient in other components of the DNA-PK pathway, Ku, and the XRCC4 protein believed to be important for recruiting ligase IV (*17*). Similar reductions were observed in infected Ku86(−) and XRCC4(−) Chinese hamster ovary (CHO) cells when compared with control CHO cells (Table 1). Such reductions are expected if the majority of the *scid*, Ku(−), and XRCC4(−) cells die because they cannot repair the integration intermediate. Increased production of cytoplasmic histone-associated DNA fragments in the infected *scid* fibroblasts suggested that they also die by apoptosis (*12*). Thus, we conclude that *scid* fibroblasts and pre-B cells respond to retrovirus infection similarly and that the efficiency of integration is reduced substantially in the absence of the three components of the DNA-PK pathway that are missing or deficient in the mutant cell lines (*17*).

We then examined whether another retrovirus could induce cell death in *scid* cells. For these experiments, we used a vesicular stomatitis virus G protein–pseudotyped human immunodeficiency virus type–1 (HIV-1) vector in which the viral genes were replaced with a *lacZ* reporter gene under control of a cytomegalovirus promoter; β-galactosidase (β-Gal) was expressed efficiently only after vector integration (*18*). Infection with this vector also induced cell death in *scid* cells (Fig. 2A), and β-Gal expres-





	% of control cells (N2 line)			% of scid cells (S33 line)		
	mock	12 hrs	16 hrs	mock	12 hrs	16 hrs
normal (N)	98	97	98	98	66	51
apoptotic (A)	2	3	2	2	33	45

Fig. 1. Viability of infected *scid* cells. (**A**) Viability of *scid* cells after infection with R/M. Cells were infected with R/M (●) at a multiplicity of ~2 i.u. per cell or with an equal amount of heat-inactivated R/M (▲) as a control. An equal volume of conditioned medium (mock-infected; ○) from uninfected cells was also used as a control. The virus stock was also diluted 1:2 (multiplicity of ~1; □) or 1:10 (multiplicity of ~0.2; △), and the viability of cells infected with these dilutions was monitored. Cells were harvested at the indicated time points, and viability was measured by trypan blue exclusion. An average of two independent counts is shown; the experiment was repeated five times with virtually identical results. Error bars represent SD. (**B**) Viability of *scid* cells after infection with an integrase-defective virus. Cells were infected with integrase-defective virus (IN^-; □) and integration-competent virus (IN^+; △) at a multiplicity of about 2 i.u. per cell. In addition, cells were infected with an equal amount of the parent, R/M virus (●). Cells were harvested at the indicated time points, and viability was measured by trypan blue exclusion. (**C**) Apoptosis in infected normal and *scid* cells. Control (N2) and *scid* (S33) cells were mock-infected or infected with the R/M virus at an MOI of ~4 i.u. per cell. Cells were harvested 12 and 16 hours later, fixed, permeabilized, labeled with the TUNEL reaction mixture (*14*), and analyzed by flow cytometry. The percentage of cells in each fraction was determined with the CellQuest program.

sion (*19*) was reduced substantially in these cells (Fig. 2B). Thus, *scid* cells also appear to undergo apoptosis in the absence of repair of the HIV-1 DNA integration intermediate. Finally, similar *scid* cell killing was also observed after infection with Moloney murine leukemia virus (*12*).

To determine if DNA-PK activity increases as a consequence of retroviral integration, we studied HeLa cells, in which DNA-PK activity can be detected readily upon stimulation by addition of damaged (sheared) DNA (*20*) (Fig. 3A). HeLa cell cultures were infected with the IN^+ or IN^- viruses, and samples were collected every 4 hours (Fig. 3B). Nuclear lysates were then tested for DNA-PK activity in the absence of added sheared DNA. A twofold to threefold increase in DNA-PK activity in IN^+ virus–infected cells occurred 8 to 16 hours after infection (Fig. 3B), whereas a negligible difference in activity was seen in IN^--infected cells. Thus, DNA-PK activity is stimulated by an early event in retroviral replication that depends on IN and appears to be sensed as DNA damage. It seems likely that the critical event is formation of the integration intermediate.

These results indicate a role for the DNA-PK–mediated repair pathway in retroviral integration, but the requirement is not absolute. Although the efficiency is greatly reduced, some stable integration is observed in Ku86(−), XRCC4(−), and DNA-PKcs(−) *scid* cells (Table 1). At least two interpretations of these results are possible; residual function of the DNA-PK pathway could account for the residual integration activity, or an alternative pathway may compensate partially for defects in the DNA-PK pathway. We note that our results do not exclude a role for viral proteins in either the primary or compensatory pathways. Although we favor the hypothesis that another pathway or pathways may compensate for deficiencies in DNA-PK, we note that of the cell lines tested, only the XRCC4(−) line is genetically null (*17*).

In the initial steps of V(D)J recombination, signal sequences direct cleavage by RAG-1 and RAG-2 proteins. In vitro studies have revealed that the RAG proteins catalyze reactions that are mechanistically similar to those carried out by retroviral IN (*21*). In V(D)J recombination, DNA-PK is required for joining of the cleaved nonhomologous DNA coding ends and is thought to interact with RAG proteins (*22*). The data presented here indicate that cellular DNA-PK–dependent DNA repair is also required to complete retroviral DNA integration. These results and the observation that both RAG and IN reactions are stimulated by HMG DNA-binding proteins (*23*) strengthen the case for evolutionary relatedness of V(D)J recombination and retroviral integration (*24*).

Although the DNA-PK pathway has been studied mainly in the context of double-strand break repair through NHEJ, our results indicate that this pathway may play a role in repairing other types of DNA lesions. In the current model for retroviral integration, the joining reaction proceeds through a concerted cleavage and ligation mechanism whereby double-strand breaks in host DNA are avoided. This may reflect evolutionary selection for minimal damage to host DNA during integration. However, the IN-catalyzed reaction forms an intermediate in which both ends of the viral DNA are flanked by short gaps in host DNA. It is possible that this structure is detected as DNA damage, as in vitro studies indicate that DNA-PK can be activated by gaps (*25*). Alternatively, a true double-strand break or some other structural feature might be responsible for triggering DNA-PK activity during integration. Whether DNA-PK plays a direct or indirect role in mobilizing the enzymatic activities that are required to complete retroviral DNA integration remains to be investigated. However, the find-

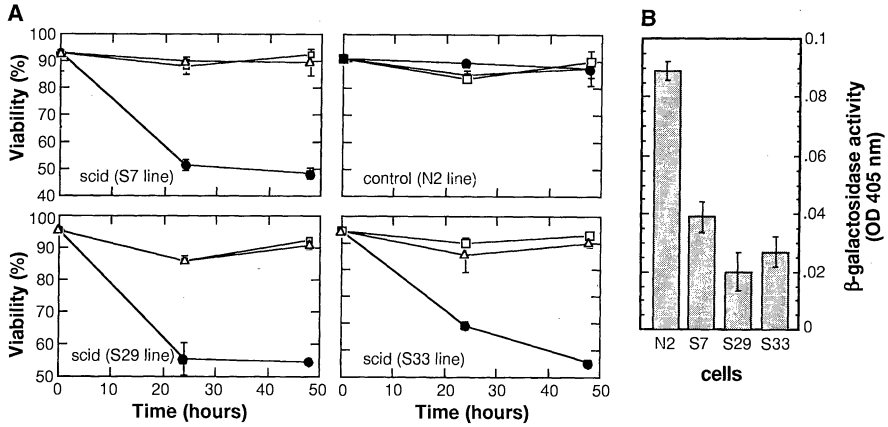


Fig. 2. Infection of *scid* cells with an HIV-1–based virus vector. (**A**) Viability of *scid* cells after infection with the HIV-1–based vector. Cells were infected under the conditions described (*18*). At the indicated time points, cells were harvested, and viability was measured by trypan blue exclusion. ●, infection with the HIV-1 vector; □, infection with heat-inactivated HIV-1 vector; Δ, mock-infected cells. (**B**) β-Gal activity in HIV vector–infected *scid* and control cells. Cells were harvested 48 hours after infection, and β-Gal assays were performed on equal numbers of viable, trypan blue–excluding cells with *o*-nitrophenyl-β-D-galactopyranoside as a substrate (*19*).

Table 1. Retroviral DNA integration in cells defective in DNA-PK components. The Ku86(−) (*xrs*-6) and XRCC4(−) cell lines have been described (*12*, *15*). These and the normal CHO cell line were obtained from D. B. Roth. All cells were plated at 10^5 per 60-mm dish and infected for 2 hours with the virus. G418 (1 mg/ml) was added 24 hours after infection, and colonies were counted 7 to 14 days later. Numbers represent the average of colonies counted on two plates for each assay. $G418^r$, G418 resistant.

Virus dilution	Number of $G418^r$ colonies in CHO cell lines		
	Normal	Ku86(−)	XRCC4(−)
10^{-3}	218	44	18
10^{-4}	17	4	1

Virus dilution	Number of $G418^r$ colonies in mouse embryo fibroblasts	
	Normal	*scid*
10^{-2}	203	24
10^{-3}	35	3

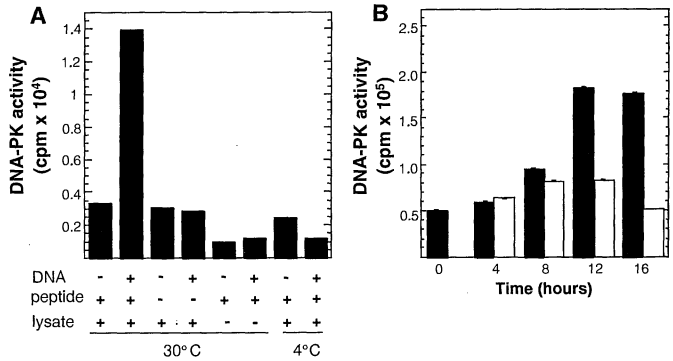


Fig. 3. DNA-PK activity in uninfected HeLa cells and avian virus vector–infected HeLa cells. (**A**) Nuclear extracts of uninfected HeLa cells were assayed for DNA-PK activity (*20*) at 30°C or 4°C, in the presence or absence of sheared salmon sperm DNA, γ^{32}P-labeled ATP, and a p53 peptide substrate. (**B**) DNA-PK activity in HeLa cells infected with IN^+ (solid bars) or IN^- (open bars) viruses at a multiplicity of ~2 i.u. per cell. DNA-PK activity in nuclear lysates was measured as in (A) except that no exogenous DNA was added.

ing that a cellular repair protein or proteins are required for this reaction may have practical ramifications. Such proteins represent a previously unrecognized set of targets for inhibition of this early step in virus replication.

References and Notes

1. P. O. Brown, in *Retroviruses*, J. M. Coffin, S. H. Hughes, E. Varmus, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1998), pp. 161–203; R. A. Katz and A. M. Skalka, *Annu. Rev. Biochem.* **63**, 133 (1994).
2. R. Craigie, T. Fujiwara, F. Bushman, *Cell* **62**, 829 (1990); R. A. Katz, G. Merkel, J. Kulkosky, J. Leis, A. M. Skalka, *ibid.* **63**, 87 (1990).
3. J. A. Gaken *et al.*, *J. Virol.* **70**, 3992 (1996).
4. P. A. Jeggo, *Adv. Genet.* **38**, 185 (1998); *Mutat. Res.* **384**, 1 (1997); S. P. Lees-Miller, *Biochem. Cell. Biol.* **74**, 503 (1996).
5. G. C. Bosma, R. P. Custer, M. J. Bosma, *Nature* **301**, 527 (1983).
6. J. S. Danska, D. P. Holland, S. Mariathasan, K. M. Williams, C. J. Guidos, *Mol. Cell. Biol.* **16**, 5507 (1996); T. Blunt *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 10285 (1996).
7. C. U. Kirchgessner *et al.*, *Science* **267**, 1178 (1995); S. P. Lees-Miller *et al.*, *ibid.*, p. 1183.
8. S7, S29, and S33 *scid* cells and N2 control cells are Abelson virus-transformed pre-B mouse cell lines described previously [W. Schuler *et al.*, *Cell* **46**, 963 (1986)].
9. E. V. Barsov and S. H. Hughes, *J. Virol.* **70**, 3922 (1996). RCASBP-M(4070A) (denoted here as R/M) is an avian retrovirus vector encoding an amphotropic envelope. Virus stocks were produced by DNA transfection and spread in chicken DF-1 cells [M. Himly, D. N. Foster, I. Bottoli, J. S. Iacovoni, P. K. Vogt, *Virology* **248**, 295 (1998)].
10. For viability measurements, suspension cells (10^6 cells per milliliter per well) were plated on 24-well plates. The multiplicity of infection (MOI) corresponds to the number of i.u. of virus per target cell. Here, an i.u. is defined by stable transduction of a reporter gene in a target cell. We measured transduction with a neo^r avian retrovirus vector, selecting for G418 resistance. Reverse transcriptase (RT) activity per i.u. was determined, and RT activity was then used to estimate the i.u. (and MOI) for each experiment with this vector and its derivatives. As a control, virus was inactivated by incubation for 30 min at 56°C. All infections were performed in the presence of diethylaminoethyl dextran (5 μg/ml).
11. To construct an IN$^-$ variant of R/M, we first subcloned a neo^r marker into the Cla I site of this vector, creating R/Mneo. To introduce the D64E-encoding mutation, we replaced an Hpa I/Asp718 fragment of R/Mneo with that of a SR-B proviral DNA carrying the IN-inactivating D64E mutation [J. Kulkosky, K. S. Jones, R. A. Katz, J. P. G. Mack, A. M. Skalka, *Mol. Cell. Biol.* **12**, 2331 (1992)], creating R/M(D64E)neo, denoted as IN$^-$ virus. A control virus was constructed by replacing an Hpa I/Asp718 fragment of R/Mneo with that of the wild-type SR-B proviral DNA, creating R/M(8KS)neo, denoted as IN$^+$ virus. Producer cells for these viruses were generated first by transfection of Q2bn helper cells [A. W. Stoker and M. J. Bissell, *J. Virol.* **62**, 1008 (1988)] with proviral DNA, followed by infection of DF-1 cells with the supernatant 3 days after transfection. DF-1 cells were selected for G418 resistance 1 day after infection. G418-resistant colonies were then pooled, and the titers of virus produced by these cells were determined by RT assay.
12. R. Daniel, R. A. Katz, A. M. Skalka, data not shown.
13. K. A. Biedermann, J. Sun, A. J. Garcia, L. M. Tosto, J. M. Brown, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1394 (1991); G. M. Fulop and R. A. Phillips, *Nature* **347**, 479 (1990); C. J. Guidos *et al.*, *Genes Dev.* **10**, 2038 (1996).
14. TUNEL labeling was performed with the In Situ Cell Death Detection Kit, Fluorescein (BMB). N2 and S33 cells were plated (0.5×10^6 cells per milliliter per well) on 24-well plates and infected with the R/M virus. All other conditions were as described (*10*).
15. Cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) were detected with the Cell Death Detection enzyme-linked immunosorbent assay kit (Boehringer Mannheim); caspase-3 activity was detected with the ApoAlertTM CPP32/Caspase-3 kit (CLONTECH). For these experiments, dexamethosone-induced apoptosis was used as a positive control, at a concentration (1 μM) reported to induce programmed death in 40% of treated cells [Y. Gavrieli, Y. Sherman, S. A. Ben-Sasson, *J. Cell Biol.* **119**, 493 (1992)].
16. Mouse embryo fibroblasts (7th passage) were plated at 1×10^5 cells per 60-mm dish and infected with serial dilutions of the virus.
17. P. A. Jeggo and L. M. Kemp, *Mutat. Res.* **112**, 313 (1983); T. D. Stamato, R. Weinstein, A. Giaccia, L. Mackenzie, *Somat. Cell Mol. Genet.* **9**, 165 (1983); N. J. Finnie, T. M. Gottlieb, T. Blunt, P. A. Jeggo, S. P. Jackson, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 320 (1995); B. K. Singleton *et al.*, *Mol. Cell. Biol.* **17**, 1264 (1997); Z. Li *et al.*, *Cell* **83**, 1079 (1995); U. Grawunder, D. Zimmer, P. Kulesza, M. R. Lieber, *J. Biol. Chem.* **273**, 24708 (1998); U. Grawunder *et al.*, *Nature* **388**, 492 (1997).
18. The HIV-1–based, VSV G protein–pseudotyped vector was described previously [L. Naldini *et al.*, *Science* **272**, 263 (1996)]. Quantitation of i.u. was based on stable transduction of the *lacZ* reporter gene, as determined by the number of blue colonies after infection of the control (N2) cell line. Cells were plated (0.5×10^6 cells per milliliter per well) on 24-well plates and then infected with 400 μl of virus per well (MOI ~2 i.u. per cell) or with virus that had been heat inactivated (56°C for 30 min). All other conditions were as described (*12*).
19. The β-Gal assay was performed according to the Transfection MBS mammalian transfection kit (Stratagene), with 5×10^5 viable cells per sample.
20. HeLa cells were plated (10^6 cells per 100-mm dish) and incubated the next day with 10 ml of virus for 2 hours. Nuclear extracts of HeLa cells were prepared as described [D. Bandyopadhyay, M. Mandal, L. Adam, J. Mendelsohn, R. Kumar, *J. Biol. Chem.* **273**, 1568 (1998)]. The p53-related peptide Glu-Pro-Pro-Leu-Ser-Gln-Glu-Ala-Phe-Ala-Asp-Leu-Trp-Lys-Lys (Promega) was used as substrate, and the kinase assay was performed as described (*25*), except that nuclear lysate (1 μl per 30 μl of reaction) was used instead of purified DNA-PK. To activate DNA-PK, we added sheared salmon sperm DNA at 100 ng per sample. The reaction was incubated for 30 min at 30°C, except for control samples, which were kept at 4°C, as indicated.
21. D. C. van Gent, K. Mizuuchi, M. Gellert, *Science* **271**, 1592 (1996).
22. D. C. van Gent, K. Hiom, T. T. Paull, M. Gellert, *EMBO J.* **16**, 2665 (1997).
23. A. Aiyar, P. Hindmarsh, A. M. Skalka, J. Leis, *J. Virol.* **70**, 3571 (1996); C. M. Farnet and F. C. Bushman, *Cell* **88**, 483 (1997); P. Hindmarsh *et al.*, *J. Virol.*, in press.
24. A. Agrawal, Q. M. Eastman, D. G. Shatz, *Nature* **394**, 744 (1998); K. Hiom, M. Melek, M. Gellert, *Cell* **94**, 463 (1998).
25. V. E. Morozov *et al.*, *J. Biol. Chem.* **269**, 16684 (1994).

26. We thank M. Bosma, R. Perry, and C. Seeger for critical comments on the manuscript; J. Kulkosky and R. Pomerantz for help with the HIV-1–based vector experiments; M. Bosma and G. Bosma for the *scid* and wild-type mouse cell lines and fibroblasts; S. Hughes for the ASV vector; I. Verma for the HIV-1 vector; D. B. Roth and M. Oettinger for helpful advice and cell lines; P. A. Jeggo for the Ku86(−) cells; and T. D. Stamato for the XRCC4(−) cells. Supported by NIH grants CA71515, AI40385, AI40721, and CA06927 and by an appropriation from the Commonwealth of Pennsylvania.

3 December 1998; accepted 16 March 1999

Nonproteolytic Neuroprotection by Human Recombinant Tissue Plasminogen Activator

Yang-Hee Kim,[1,2] June-Hee Park,[1] Seung Hwan Hong,[2] Jae-Young Koh[1]*

Human recombinant tissue plasminogen activator (tPA) may benefit ischemic stroke patients by dissolving clots. However, independent of thrombolysis, tPA may also have deleterious effects on neurons by promoting excitotoxicity. Zinc neurotoxicity has been shown to be an additional key mechanism in brain injuries. Hence, if tPA affects zinc neurotoxicity, this may provide additional insights into its effect on neuronal death. Independent of its proteolytic action, tPA markedly attenuated zinc-induced cell death in cortical culture, and, when injected into cerebrospinal fluid, also reduced kainate seizure–induced hippocampal neuronal death in adult rats.

Human recombinant tPA can benefit stroke patients by lysing clots (*1*). However, recent evidence suggests that it may also harm neurons by promoting excitotoxic injury (*2*). This detrimental effect of tPA may be mediated by plasmin, the main product of tPA action in thrombolysis, because not only tPA-null but also plasminogen-null mice are resistant to excitotoxic injury (*3*).

Although excitotoxicity is a key mechanism of pathologic neuronal death in many cases (*4*), neurotoxicity mediated by endogenous zinc translocation has recently been shown to be another major mechanism of selective neuronal death in global isch-

[1]National Creative Research Initiative Center for the Study of Central Nervous System Zinc and Department of Neurology, University of Ulsan College of Medicine, 388-1 Poongnap-Dong Songpa-Gu, Seoul 138-736, Korea. [2]Department of Molecular Biology and Institute for Molecular Biology and Genetics, Seoul National University, Seoul, Korea.

*To whom correspondence should be addressed. E-mail: jkko@www.amc.seoul.kr