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DNA-PK is essential only for coding joint formation in V(D)J recombination

Piotr Kulesza and Michael R. Lieber*

Department of Pathology, Department of Biochemistry and Molecular Biology and Department of Molecular Microbiology and Immunology, Norris Comprehensive Cancer Center, Room 5425, University of Southern California School of Medicine, 1441 Eastlake Avenue, Los Angeles, CA 9003, USA

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ABSTRACT

The analysis of the role of DNA-dependent protein kinase (DNA-PK) in DNA double-strand break repair and V(D)J recombination is based primarily on studies of murine scid, in which only the C-terminal 2% of the protein is deleted and the remaining 98% is expressed at levels that are within an order of magnitude of normal. In murine scid, signal joint formation is observed at normal levels, even though coding joint formation is reduced over three orders of magnitude. In contrast, a closely associated protein, Ku, is necessary for both coding and signal joint formation. Based on these observations, a reasonable hypothesis has been that absence of the DNA-PK protein (rather than merely its C-terminal 2% truncation) would ablate signal joint formation along with coding joint formation. In fact, a study of equine SCID, in which there is a much larger truncation of the DNA-PK protein, has suggested that signal joints do fail to form. In our current study, we have analyzed signal and coding joint formation in a malignant glioma cell line, M059J, which was previously shown to be deficient in DNA-PK. Our quantitative analysis shows that full-length protein levels are reduced at least 200-fold, to a level that is undetectable, yet signal joint formation occurs at wild-type levels. This result demonstrates that at least this form of non-homologous DNA end joining can occur in the absence of DNA-PK.

INTRODUCTION

One of the most damaging effects of ionizing radiation is generation of DNA double-strand breaks. Mammalian cells have evolved pathways to cope with this type of lesion and some insight has been gained into the mechanism and the proteins required for DNA end joining. Most of the information stems from analysis of rodent cell lines hypersensitive to ionizing radiation. These can be subdivided into at least seven X-ray cross-complementing groups (XRCC), of which four show a deficiency in end joining. The *XRCC5* gene encodes Ku86 (1,2). Ku86 forms a heterodimer with the Ku70 polypeptide (encoded by *XRCC6*) (3) and this complex associates with DNA termini. *XRCC4* encodes a 38 kDa protein (4) which stimulates the activity of DNA ligase IV (5). *XRCC7* encodes the 470 kDa DNA-dependent protein kinase (DNA-PK) (6,7). Expression of human DNA-PK restores wild-type resistance to ionizing radiation in murine scid cells (8) and it has been shown that the murine scid *DNA-PK* gene has an ochre mutation which results in a truncated protein (9–12). Another mutation has been identified in equine SCID, where a deletion results in premature polypeptide termination upstream of the kinase domain of DNA-PK (13). All cell lines deficient in DNA-PK activity exhibit increased sensitivity to radiation damage and a decreased capacity for double-strand break repair (DSBR) (14–16). In addition, both scid mice and SCID foals show profound immunodeficiency due to lack of development of B and T cells (15,17).

V(D)J recombination, the process responsible for generation of antigen receptors in B and T cells, is dependent on lymphoid-specific recombination activation genes 1 and 2 (*RAG*-1 and *RAG*-2) (18), as well as ubiquitously expressed DSBR components. RAG proteins recognize and cleave DNA at recombination signal sequences (RSS) (19), which are adjacent to the coding regions of immunoglobulin and T cell receptor (TCR) genes. RAG-mediated cutting results in blunt double-stranded ends at the RSS and hairpinned coding ends, which are then processed and rejoined by other proteins. All the above gene products involved in DSBR also play a role in V(D)J recombination. Deficiencies in XRCC4 and the Ku 70/86 complex result in a lack of formation of both signal and coding joints. Mutations in DNA-PK uniformly block the formation of coding joints, most likely due to lack of hairpin opening (20), but signal joint formation is less severely affected. Studies of Chinese hamster ovary cell line V3 and cell lines derived from scid mice show normal levels of signal joint formation, with a fraction of signals ends joined imprecisely (21–24). However, a marked reduction in the level of signal joint formation has been found in equine SCID fibroblasts (15). Hence, two studies in which DNA-PK was thought to be absent (V3 and equine SCID) yielded different results concerning signal joint formation.

The expression of DNA-PK protein is different in the studied mutant cells. Murine scid cells were found to express a mutant DNA-PK protein, albeit at a lower level, whereas expression was not detected in V3 and equine SCID fibroblasts (7,9,15). However, it is important to note that anti-human monoclonal antibodies were used to detect DNA-PK in these rodent and equine cells and it is possible that the sensitivity of detection was very low. The

*To whom correspondence should be addressed. Tel: +1 323 865 0568; Fax: +1 323 865 3019; Email: lieber_m@froggy.hsc.usc.edu

sensitivity levels could have been determined by performing titrations of the control cell extracts by western blotting. However, this was not done and in the absence of this information, no conclusion can be made about the magnitude of reduction in DNA-PK protein expression in V3 or equine SCID cells. This poses a problem in interpretation of the V(D)J recombination results, since low levels of protein could be responsible for the wild-type levels of signal joint formation in V3 cells, possibly reconciling the difference between V3 and equine SCID.

In order to address the issue of expression levels quantitatively and to provide additional insight into whether DNA-PK is or is not required for signal joint formation, a human malignant glioma cell line shown to be deficient in expression and activity of DNA-PK was analyzed in this study (25).

MATERIALS AND METHODS

Cell lines

The malignant glioma lines M059J (DNA-PK-deficient mutant) and M059K (wild-type) were a kind gift of Dr J. Allalunis-Turner (16). Cells were grown in RPMI medium supplemented with 10% fetal calf serum, 1 mM glutamine and 5 µM 2-mercaptoethanol under standard tissue culture conditions. Cells were harvested by trypsinization, washed in phosphate-buffered saline and either used for transfections or frozen as pellets for protein and RNA analysis.

Protein analysis

For western blotting, cell pellets were lysed in RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM DTT, pH 8.0) supplemented with protease inhibitors leupeptin, pepstatin, aprotinin, antipain (all at 1 µg/ml) and PMSF (at 1 mM). After brief centrifugation, the protein concentration of the supernatants was determined using bicinchoninic acid reagent (BCA; Pierce). These extracts were then diluted to 10 mg/ml, boiled in sample buffer, diluted further and analyzed by Tris-buffered SDS–PAGE. After transfer to nitrocellulose, blots were probed with monoclonal antibodies 18-2, 25-6 and 42-27 for DNA-PK (26) or D6D9 for Ku70 (27). A goat anti-mouse horseradish peroxidase-conjugated secondary antibody was then used and the signal was developed using ECL (Amersham).

RNA analysis

Total RNA was extracted from cell pellets using RNA-zol (Tel-Test) and the concentration determined following resuspension in DEPC-treated water. Northern blotting was performed as previously described (28), with 20 µg RNA loaded per lane. Probes were generated by random priming using templates obtained by PCR with primers indicated in the legend to Figure 2. HPRT primer sequences were as follows: antisense, GCTTTTCCAGTTTCACTAATGACA; sense, TGGGAGGCCATCACATTGT. First strand cDNA synthesis was performed under standard conditions using M-MLV reverse transcriptase (Gibco-BRL). For cDNA used to amplify the 3′-region of *DNA-PK* and *HPRT*, reactions were primed with a poly(dT) 18mer; for cDNA used for amplification of the 5′-region, reactions were primed with primer complementary to the coding sequence of *DNA-PK* from base 5545 to 5580. For PCR, primers identical to the *DNA-PK* coding sequence at bases 634–656 (sense strand) and 2401–2377 (antisense strand) were used to amplify the 5′-region, primers identical to the *DNA-PK* coding sequence at bases 9587–9631 (sense strand) and 12384–12356 (antisense strand) were used to amplify the 3′-region. The conditions for PCR were as follows: 96°C for 1 min, 55° C for 1 min, 72°C for 4.30 min for 30 cycles.

V(D)J recombination assays

Cells were transfected by electroporation with plasmids encoding human *RAG*-1 (pHR 1) and *RAG*-2 (pHR 2) (29), along with recombination substrate vector either pGG49 (signal joint analysis substrate) or pGG51 (coding joint analysis substrate) at a ratio of 3:3:1 and cultured for 48 h. Recombination substrates, the recovery of plasmid DNA from transfected cells and quantitation of recombination were performed as previously described (30). In brief, the pGG49 substrate bears 12 and 23 spacer recombination signals in an orientation such that V(D)J recombination results in formation of a signal joint that is retained on the plasmid. The pGG51 substrate has a 12 and 23 signal orientation such that a coding joint is retained on the plasmid. Only *Dpn*I-resistant plasmid DNA was used for *Escherichia coli* transformation. Bacterial colonies recovered on ampicillin/chloramphenicol plates were picked and grown in LB medium under selection to confirm resistance. Plasmid DNA of individual recombinants was analyzed by restriction enzyme digestion.

RESULTS

No expression of DNA-PK protein in M059J cells

In order to quantitate the level of DNA-PK deficiency, a dilutional analysis of protein expression in M059J versus M059K cell lines was performed (Fig. 1A and B). A control western blot probed for Ku70 indicated that the amount of total protein in the extracts and the dilutions were equivalent between the two cell lines (Fig. 1B). For the detection of DNA-PK, three anti-human monoclonal antibodies were used (31). The western blot signal for DNA-PK from equivalent amounts of cell extract is shown in lanes 1 and 7 (Fig. 1A). A comparison between lanes 6 and 7 (Fig. 1A) reveals that expression of DNA-PK in the mutant cell line is not detectable compared with a 1:200 dilution of the wild-type sample. In addition, no smaller fragments of DNA-PK were detectable. We conclude that the steady-state level of DNA-PK protein is reduced >200-fold and, if present at all, could be much lower. In addition, this analysis indicates that the absence of the 470 kDa DNA-PK polypeptide does not, in itself, result in any detectable decrease in the stability of Ku70 protein.

Marked reduction in DNA-PK mRNA in M059J cells

Given the absence of DNA-PK protein in M059J cells, the levels of DNA-PK mRNA were next determined in order to further define the defect. The results of northern blot analysis are shown in Figure 2. Individual strips containing wild-type and mutant cell line total RNA were analyzed with probes specific to regions of the DNA-PK mRNA sequence. The probes spanned the sequence from 5′ to 3′ in four intervals of 2.7, 2.8, 2.9 and 2.8 kb and are identified as A–D respectively (Fig. 2). The full-length message as expressed in the wild-type cells is shown in all four strips. There were no full-length or truncated DNA-PK mRNA species detectable in the mutant cell line M059J. We conclude that the mRNA level is reduced to levels that were not detectable in the northern blots.

In order to enhance the sensitivity of mRNA detection, RT–PCR analysis of the two cell lines was performed. Successive

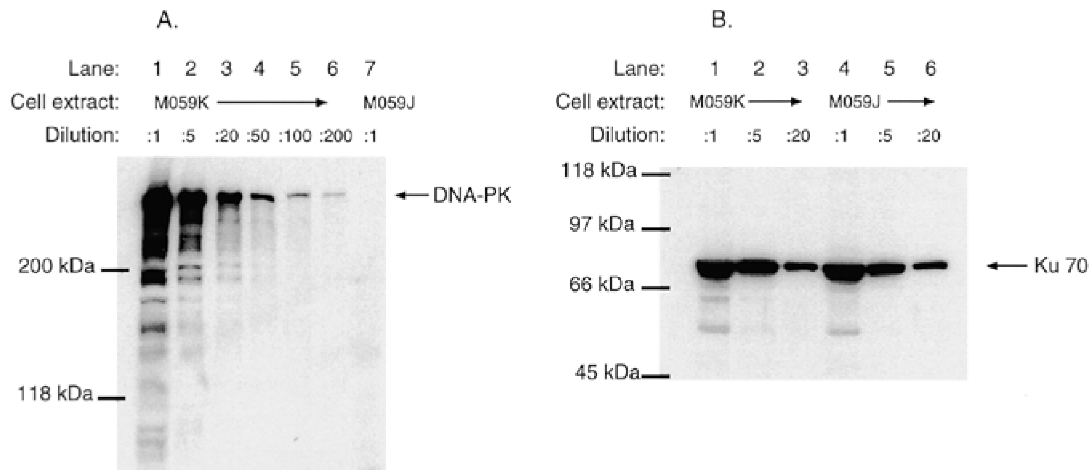

Figure 1. Quantitative analysis of DNA-PK protein expression in M059J and M059K cell lines. (**A**) Western blot analysis of cell extracts using antibodies against DNA-PK. Lanes 1–6, M059K extract at 1:1 (lane 1), 1:5 (lane 2), 1:20 (lane 3), 1:50 (lane 4), 1:100 (lane 5) and 1:200 (lane 6) dilutions; lane 7, M059J extract at a 1:1 dilution. The blot was intentionally overexposed to reveal the weakest signals. (**B**) Control western blot probed with anti-Ku70 antibody. Lanes 1–3, M059K extract at 1:1 (lane 1), 1:5 (lane 2) and 1:20 (lane 3) dilutions; lanes 4–6, M059J extract at corresponding dilutions.

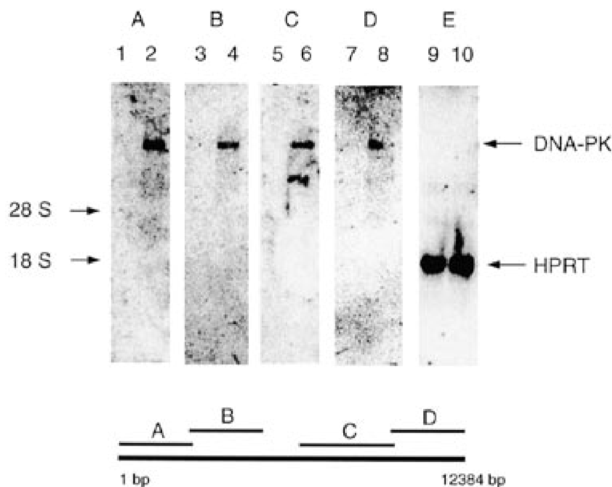

Figure 2. Analysis of DNA-PK mRNA expression in M059J and M059K cell lines using northern blotting. Lanes 1, 3, 5, 7 and 9, M059J RNA; lanes 2, 4, 6, 8 and 10, M059K RNA. The PCR-generated probe templates spanned bases 79–2750 (strip A), 2739–5545 (strip B), 6640–9624 (strip C) and 9587–12384 (strip D). Location of probes with regard to *DNA-PK* sequence is indicated by bars. A probe against *HPRT* was used in strip E. The DNA-PK blot is intentionally overexposed to demonstrate the absence of a DNA-PK mRNA band in M059J.

1:4 dilutions of cDNA were used as templates for the PCR reactions (Fig. 3). Reactions using primers designed to amplify products at the extreme 5′- and 3′-ends of the sequence were analyzed on agarose gels for product intensity and size. For the 5′-specific fragment, reactions using cDNA primed with a *DNA-PK*-specific oligonucleotide as template were performed with a primer pair designed to yield a 1.77 kb product (Fig. 3A). For the 3′-specific fragment, reactions using oligo(dT)-primed cDNA as template were performed with a primer pair which results in a 2.8 kb product (Fig. 3B) and with *HPRT* primers as a control for the amount of template cDNA (Fig. 3C). A comparison between the RT–PCR signals generated from the M059J and M059K cell lines revealed that the level of DNA-PK-specific RNA transcripts in the mutant cell line was reduced by 16–64-fold (Fig. 3A and B). Hence, at least part of the basis for the reduction in protein level appears to be a reduction in steady-state mRNA level.

Wild-type level of signal junctions in M059J cells

Given that the DNA-PK-deficient M059J cell line is radiation-sensitive and deficient in DSBR, the V(D)J recombination phenotype of this cell line was analyzed and compared with its wild-type counterpart. The extrachromosomal V(D)J recombination assay, which utilizes V(D)J recombination substrates bearing signal sequences arranged to allow signal and coding joint formation, was used for the analysis. Because the glioma cell lines do not express RAG-1 and RAG-2 proteins, expression vectors encoding these proteins were transfected along with signal and coding joint substrates to permit V(D)J recombination to initiate. Eight transfections of M059K and M059K were performed with each recombination substrate. The M059K cell line exhibited a level of coding joint formation in the range of other human cell lines wild-type for V(D)J recombination. The M059J cell line, however, showed a complete lack of ability to form coding joints (Table 1A). Both cell lines were capable of signal joint formation (Table 1B). Remarkably, the level of activity in the M059J cell line was undiminished compared with the wild-type control. The fidelity of signal joints produced by both the M059J and M059K cell lines was further analyzed by *Apa*LI digestion (Table 1B). This was possible because the precise joining of two heptamers of the signal sequence creates a new *Apa*LI site. All 44 joints recovered from the M059K cell line were precise. Seventy out of 74 (95%) signal joints recovered from the M059J cell line were precise. We conclude that the DNA-PK-deficient cell line is capable of wild-type levels of signal joint formation with a high degree of fidelity.

DISCUSSION

The current study shows that although the DNA-PK protein is not detectable and is reduced at least 200-fold in the M059J human glioma cell line, the cells are capable of signal joint formation at the wild-type level. These results strongly indicate that the entire

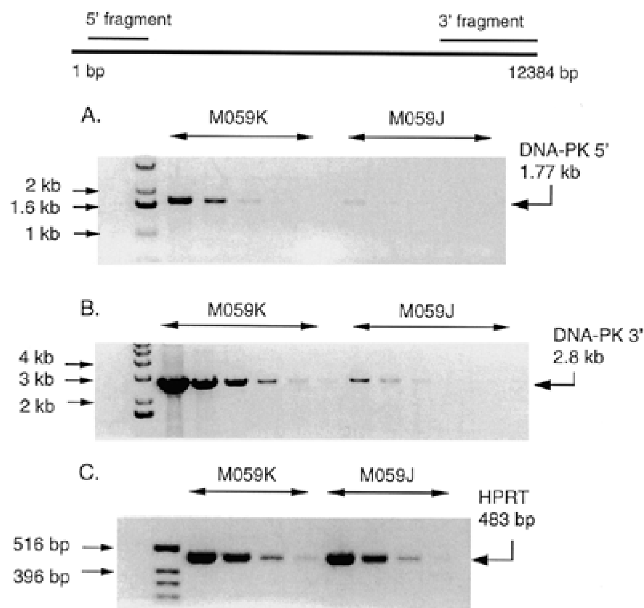

Figure 3. Analysis of DNA-PK mRNA expression in M059J and M059K cell lines using RT–PCR. Panels represent PCR reactions using cDNA templates derived from M059J or M059K cells. Bars below (C) show locations of DNA-PK PCR products. (**A**) 5′-Fragment. Template cDNA was generated using a primer specific for *DNA-PK*. Template cDNA from each of the two cell lines was serially diluted 1:4. Five reactions per cell line were set up and the products were loaded on the gel in order from no template dilution to highest template dilution. Thus, the left-most lane for each of the two cell lines corresponds to the reaction with undiluted template, while the right-most lane for each of the two cell lines corresponds to the 1:256 dilution of template. (**B**) 3′-Fragment. Template cDNA was generated using oligo(dT). Template cDNA dilutions were as described for (A) except that six reactions were set up and the right-most lane corresponds to a 1:1024 dilution of template. (**C**) Control. Template cDNA used for amplification of the 3′-fragment was serially diluted as described for (A), except that only four reactions were used. *HPRT*-specific primers were used to generate the PCR product. In all three panels the expected size of the specific product is indicated by the arrow on the right side of the gel. The lane left of the molecular weight standards represents buffer control reactions. There were no PCR products in reactions using genomic DNA as template (data not shown).

Table 1. V(D)J recombination in M059K versus M059J cells

A. Coding joint formation

Plasmid	Cell line	DpnIAmp^r	DpnIAmp^{r}Cam^r	R(%)[a]
pGG 51	M059K	80000	91	0.114
	M059J	117290	0	<0.001

B. Signal joint formation

Plasmid	Cell line	DpnIAmp^r	DpnIAmp^{r}Cam^r	R(%)[a]	Fidelity[b]
pGG 49	M059K	127960	240	0.19	100%
	M059J	140340	349	0.25	95%

[a]R(%) indicates recombination level expressed as a ratio of ampicillin + chloramphenicol-resistant colonies (Amp^{r}Cam^r) to ampicillin-resistant colonies (Amp^r).
[b]As determined by *Apa*LI digestion.

DNA-PK protein is required only for the coding joint part of the V(D)J recombination reaction. This extends in a significant way our understanding of DNA-PK, because the murine scid variant is only lacking the C-terminal 2% of the protein and analyses of V3 and equine SCID cells were unable to quantitate the level of DNA-PK protein level reduction.

The results of this study quantitatively determine the deficit in DNA-PK protein and RNA levels in the M059J cell line. No full-length DNA-PK protein products were detectable in M059J cell extracts in analysis of protein expression. Analysis of the M059J cell mRNA revealed a marked reduction in the level of the DNA-PK transcript compared with the wild-type counterpart. This decrease in the level of mRNA could be due to mutations in the regulatory sequences or suppression of transcription by methylation (32). Regardless of the mechanism of the mRNA decrease, it appears that the protein product is not expressed, since full-length or partial length DNA-PK expression in M059J was undetectable compared with even a 200-fold dilution of the M059K wild-type cell protein extract.

It is interesting to compare the recombination phenotype of the M059J cell line with that of murine scid. Murine scid and Chinese hamster ovary cell lines deficient in DNA-PK retain the ability to form correct signal joints, albeit with lower precision, in the extrachromosomal V(D)J recombination assay (21,23,24). In a different set of studies analyzing the recombination intermediates in rearrangement of the TCR δ locus in mouse thymocytes, two products with regard to signal joint formation were detected: excised linear fragments containing RSS and reciprocal circular molecules resulting from joining of the excised fragments (33). The circular products were detected in both wild-type and scid cells, indicating that the signal joining activity is present in the mutant cells (20). The murine scid defect has been defined as an ochre mutation which results in a C-terminal 83 amino acid truncation in DNA-PK. This mutant of DNA-PK lacks kinase activity. However, almost the entire length of the protein is expressed, albeit at lower levels, in scid lymphocyte precursors (9). This left open the question of whether the truncated DNA-PK molecule, which contains 98% of the total sequence, was necessary for signal joint formation. On the basis of our results, it is clear that the entire molecule is not needed for signal joint formation.

The above studies can be contrasted with the results obtained in equine SCID fibroblasts, where a 5 nt deletion in the coding sequence results in a C-terminal truncation of the last 25% of the protein (967 amino acids). Like the M059J cell line, these cells were incapable of coding joint formation. In contrast, the equine SCID cells appeared to have a reduced ability to generate signal joints (13). It is possible that the disparity between equine SCID and M059J cells could be a consequence of different experimental systems used to assay for signal joint formation. In the current study, the M059 cell lines were analyzed using recombination substrates and *RAG* expression vectors which replicate in human cells, which are likely to permit a much higher level of recombination activity. Moreover, definitive quantitative analysis and normalization for transfection efficiency could be performed, since replication of the V(D)J recombination substrate is necessary to reliably determine how much of the substrate entered the nuclei of transfected cells. In contrast, non-replicating substrates and *RAG* vectors were used in the studies of equine cells and the detection of recombinants was performed by a nested PCR analysis using two sets of cycles. It is possible that the

recombination activity in wild-type equine cell lines was very low and, therefore, the PCR-detectable signal represents a single recombination event. The authors comment that in one out of six experiments, signal junctions were detected in the mutant cell line, suggesting that a low level of signal joint formation does occur in the mutant equine cells (15). This could indicate that there is a reduction, not an absence, of signal joint formation in equine SCID fibroblasts. An alternative possibility is that the mutation in equine SCID results in a protein that is made but which functions as an inactive inhibitor of signal joint formation. The possibility of dominant negative mutants of *DNA-PK* is also raised by the analysis of a *DNA-PK* point mutant which results in ablation of coding joints and a mild (<10-fold) reduction in signal joints and significant nucleotide loss at many of the signal joints (34), as is observed in murine scid (21). Our study here indicates that the complete absence of DNA-PK (rather than its mutation or truncation) has no effect on signal joint formation level and only a mild effect on signal joint fidelity. Hence, it is possible that the presence of a defective DNA-PK protein results in more serious effects than its complete absence, as examined here.

It is possible that signal joint formation is a part of a V(D)J recombination-specific DSBR pathway which does not rely on DNA-PK. One possibility is that the RAG proteins maintain the proximity of the two signal ends to each other to facilitate ligation and, in some way, make DNA-PK unnecessary. Second, there are independent lines of evidence that some types of non-homologous DNA end joining do not require DNA-PK. Murine scid cells were shown to have varying levels of DSBR activity depending on the stage of the cell cycle (35). The DSBR activity was reduced in G_1 and early S phases, but during the rest of the cell cycle it was the same as in wild-type cells, indicating that a DNA-PK-independent mechanism could be active at particularly apparent levels in late S, G_2 and M phases. This S, G_2, M alternative pathway may be due to homologous recombination and may not be relevant to V(D)J recombination, in which the cleavage phase occurs during G_1 (36). Third, studies using murine scid fibroblasts distinguished between heat-labile and heat-resistant pathways of DNA end joining and suggested that the DNA-PK-dependent repair pathway is heat-resistant, while the alternative, DNA-PK-independent pathway is heat-sensitive (37). Finally, it has recently been shown that the kinase activity of the DNA-PK:Ku:DNA complex is heat-sensitive and that Ku, but not DNA-PK itself, is responsible for this lability (38). This points to the existence of a DNA-PK-independent, Ku-dependent heat-sensitive pathway which may be sufficient for the resolution of signal ends. The results of the current study suggest that this heat-sensitive activity may be responsible for the wild-type signal joint levels observed in the M059J human glioma cell line.

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