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Backup Pathways of NHEJ Are Suppressed by DNA-PK

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Abstract In cells of higher eukaryotes double strand breaks (DSBs) induced in the DNA after exposure to ionizing radiation (IR) are rapidly rejoined by a pathway of non-homologous end joining (NHEJ) that requires DNA dependent protein kinase (DNA-PK) and is therefore termed here D-NHEJ. When this pathway is chemically or genetically inactivated, cells still remove the majority of DSBs using an alternative, backup pathway operating independently of the *RAD52* epistasis group of genes and with an order of magnitude slower kinetics (B-NHEJ). Here, we investigate the role of DNA-PK in the functional coordination of D-NHEJ and B-NHEJ using as a model end joining by cell extracts of restriction endonuclease linearized plasmid DNA. Although DNA end joining is inhibited by wortmannin, an inhibitor of DNA-PK, the degree of inhibition depends on the ratio between DNA ends and DNA-PK, suggesting that binding of inactive DNA-PK to DNA ends not only blocks processing by D-NHEJ, but also prevents the function of B-NHEJ. Residual end joining under conditions of incomplete inhibition, or in cells lacking DNA-PK, is attributed to the function of B-NHEJ operating on DNA ends free of DNA-PK. Thus, DNA-PK suppresses alternative pathways of end joining by efficiently binding DNA ends and shunting them to D-NHEJ. J. Cell. Biochem. 92: 781–794, 2004.

Key words: nonhomologous end-joining (NHEJ); double strand breaks (DSB); DNA-PK; wortmannin

Double strand breaks (DSBs) induced in the DNA by endogenous processes or exogenous agents such as ionizing radiation (IR) undermine genomic integrity and can, in principle, be removed by homology directed repair (HDR) or non-homologous end joining (NHEJ) [Haber, 1999; Doherty and Jackson, 2001; Thompson and Schild, 2001; Jackson, 2002]. In cells of higher eukaryotes, repair of IR induced DNA DSBs is dominated by a fast component operating with half times of the order of a few minutes. This form of rejoining is severely compromised by defects in any of the constituents of DNA-PK, DNA-PK catalytic subunit (DNA-PKcs), and Ku heterodimer [Nevaldine et al., 1997; DiBiase et al., 2000a; Wang et al., 2001b], as well as in cells defective in DNA ligase IV [Wang et al., 2001c]. These proteins operate in the same pathway of NHEJ, which we proposed the term D-NHEJ, to indicate its dependence on DNA-PK.

Cells with defects in DNA-PK activity, or the DNA ligase IV complex are nevertheless able to rejoin the majority of IR-induced DNA DSBs utilizing an alternative pathway operating with 20–30 fold slower kinetics [Nevaldine et al., 1997; DiBiase et al., 2000b; Wang et al., 2001c]. The slower kinetics of this component of DNA DSB rejoining is compatible with the operation of HDR. We tested this hypothesis with the hyper-recombinogenic DT40 chicken cell line and mutants defective in homologous recombination (HR) [Sonoda et al., 1998; Takata et al., 1998]. DT40 cells rejoin IR-induced DNA DSBs with kinetics similar to those of other vertebrate cells despite the 1,000-fold increase in HR [Wang et al., 2001b]. In addition, knockouts of RAD51B, RAD52, and RAD54 rejoin DNA DSB

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with kinetics similar to the wild type, as does also a conditional knock out mutant of RAD51 [Wang et al., 2001b]. Finally, a double mutant $Ku70^{-/-}/RAD54^{-/-}$ shows similar half times to $Ku70^{-/-}$ cells [Wang et al., 2001b]. On the basis of these observations and because the slow component of DNA DSBs rejoining is error prone [Loebrich et al., 1995], we hypothesized that it reflects an alternative pathway of NHEJ operating as a backup, and which we therefore proposed to refer to as B-NHEJ—to differentiate from D-NHEJ. According to this model, at least two distinct NHEJ pathways cooperate to remove IR-induced DNA DSBs from the genome of higher eukaryotes [Asaad et al., 2000; DiBiase et al., 2000b; Wang et al., 2001b].

We inquired whether biochemical studies utilizing as substrate plasmid DNA digested by a restriction endonuclease to generate different types of ends, and cellular extracts as a source of enzyme, can recapitulate aspects of the operation of the two pathways of DNA DSB rejoining. Here, we examine this possibility by studying the role of DNA-PKcs in NHEJ using an in vitro plasmid-based DNA end joining assay.

MATERIALS AND METHODS

Cell Lines and Extract Preparation

HeLa cells were grown either as monolayer or suspension cultures in Joklik's modified MEM (S-MEM) supplemented with 5% bovine calf serum. M059J cells were grown as monolayer cultures in DMEM/F12 medium supplemented with 10% bovine calf serum. Experiments were performed either with whole cell extract (WCE) or with nuclear extract (NE). For the preparation of HeLa cell extracts a 1–30 L suspension of cells was grown in spinner flasks to $0.5–1 \times 10^6$ cells/ml and collected by centrifugation. For M059J extract-preparation, 100 dishes (100 mm, 20 ml growth medium, 0.5×10^6 cells) were prepared and cells were allowed to grow to approximately 4×10^6 cells/dish before harvesting by trypsinization. The slow growth characteristics of M059K cells, the isogenic wild type control of M059J cells, made extract preparation difficult. Therefore, in this study HeLa cells are used as a wild type control for M059J cells. For further processing, cells from both cell lines were washed once in ice-cold PBS and subsequently in five packed cell volumes of cold hypotonic buffer (10 mM Hepes, 5 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM phenylmethylsulfonyl fluoride, PMSF, and 0.5 mM DTT). The cell pellet was resuspended in two volumes of hypotonic buffer and, after 10 min in ice, disrupted in a Dounce homogenizer (15 strokes with a "B" pestle). For the preparation of WCE, 3 M KCl was slowly added to the homogenate to a final concentration of 0.5 M KCl and, after 30 min on ice, centrifuged for 40 min at 14,000 rpm at 4°C. To remove DNA from the extract, one tenth extract volume of DEAE Sepharose, equilibrated in dialysis buffer (25 mM Hepes, pH 7.5, 100 mM KCl, 1 mM EDTA, 10% glycerol, 0.2 mM PMSF, and 0.5 mM DTT), was added and the mixture was gently rotated at 4°C for 30 min. Extract was cleared by centrifugation at 12,000 rpm for 5 min and dialyzed overnight in dialysis buffer. After centrifugation, aliquots of the extract were snap frozen and stored at −80°C.

For NE preparation cells were homogenized as above and 3 M KCl was slowly added to a final concentration of 50 mM KCl. The extract was incubated for 10 min on ice and centrifuged for 30 min at 3,300*g* at 4°C. Supernatant was collected as cytoplasmic extract (CE). Nuclear pellet was resuspended in two packed nuclear volumes (pnv) of low salt buffer (20 mM Hepes, pH 7.9, 20 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT) and 1 pnv of high salt buffer (10 mM Hepes, pH 7.9, 1.6 M KCl, 1.5 mM $MgCl_2$) was slowly added to a final concentration of 400 mM KCl. Extract was incubated for 30 min at 4°C under gentle rotation and centrifuged for 30 min at 50,000*g* at 4°C. The supernatant was collected as NE. In some preparations of NE, DEAE Sepharose (0.1 volume) was added to remove DNA as described above for WCE and dialyzed overnight in dialysis buffer (20 mM Hepes, pH 7.9, 10% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT) before snap freezing and storing aliquots. Alternatively, the DEAE step was omitted and NE was dialyzed in buffer containing 400 mM KCl. For the purposes of the present study both methods of NE preparation gave extracts with comparable activities, although variations were observed from preparation to preparation.

Proteins and Antibodies

DNA-PKcs and DNA-PK were purified from HeLa nuclear extracts as previously described [Hartley et al., 1995]. Antibodies against DNA-PKcs (Ab-2) were purchased from Neomarkers (Union City, CA).

Plasmid Preparation and DNA End Joining

Supercoiled plasmid pSP65 (3 kb; Promega, Mannheim, Germany) was prepared using $CsCl_2$/EtBr gradients. It was used as a substrate in DNA end joining reactions after digestion with *Sal* I to generate linearized DNA. In some experiments, pSP65 was linearized using other restriction endonucleases as indicated.

End joining reactions were performed in 20 mM Hepes-KOH (pH 7.5), 10 mM $MgCl_2$, 80 mM KCl, 1 mM ATP, 1 mM DTT, 0.25 μg of DNA (12.5 ng/μl), and 0–20 μg (0–1 μg/μl) of HeLa, or M059J cell extracts (NE or WCE) in a final volume of 20 μl at the indicated temperatures and for the indicated periods of time. In standardization experiments the concentrations of certain components was varied as indicated. Reactions were terminated by adding 2 μl of 5% SDS, 2 μl of 0.5 M EDTA, and 1 μl of proteinase E (10 mg/ml), and were then incubated for 1 h at 37°C. One half of the reaction was loaded on a 0.7% agarose gel and run at 45 V (2 V/cm) for 5 h. Gels were stained in 20,000× diluted SYBR Gold (Molecular Probes, Eugene, Oregon) for 1 h and scanned in a Typhoon (Amersham Biosciences, Freiburg, Germany) using a laser emitting at 532 nm with at 610 BP emission filter. Scanning was carried out at +3 mm focal plane, at normal sensitivity using 200 μm pixel size and 600 V photomultiplier voltage. The ImageQuant software (Molecular Dynamics) was used to calculate the percent of input plasmid found in dimers and other higher order polymers. The values obtained were corrected for signal observed in the same region of the gel in reactions assembled without extract. The percent end joining calculated in this way is shown at the bottom of the gel images.

Wortmannin (Sigma, Mannheim, Germany) was prepared in DMSO at 10 mM and diluted in 10% DMSO to 20 μM immediately before use. This stock was further diluted appropriately to achieve the indicated final concentrations after addition of 1 μl to the reactions, which were incubated for 10 min at 25°C prior to the addition of ATP and DNA. This pre-treatment and sequence of reagent addition was found to maximize the effect of wortmannin (see below).

Assay of DNA-PK Activity

The assay used to measure DNA-PK activity has been described [Lees-Miller et al., 1992]. Briefly, the peptide EPPLSQEAFADLWKK, corresponding to 11–24 amino acids of human p53 with threonine18 and serine 20 changed to alanine was used as substrate. Cell extracts are mixed with 200 μM peptide, with or without 10 μg/ml sonicated calf thymus DNA in 50 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 50 mM KCl, 0.2 mM EGTA in the final volume of 18 μl. A radioactively labeled ATP solution is made by mixing 1 μl of γ-[^{32}P]-ATP (3,000 Ci/mmol, 10 μCi/μl) in 49 μl unlabeled 5 mM ATP. Reactions are started by adding 2 μl of ATP solution and incubating at 30°C for 30 min; they are stopped by adding 20 μl stop solution (30% acetic acid, 1 mM ATP). Subsequently, 20 μl of the mixture is spotted on Whatman p81 phosphocellulose paper and washed four times in 10% acetic acid for 10 min. ^{32}P incorporation is measured in a Scintillation Counter (Packard, Shelton, CT). Results are analyzed to calculate net incorporation of ATP (in pmole) by subtracting incorporation in reactions assembled without DNA (background).

RESULTS

Optimization of Reaction Conditions

To investigate the interplay between D-NHEJ and B-NHEJ it was necessary to optimize the reaction conditions. Suboptimal reaction conditions, or conditions forcing DNA end joining to a single pathway [Baumann and West, 1998; Lee et al., 2000; Huang and Dynan, 2002] can lead to biased conclusions. Contrary to results previously obtained in an assay utilizing agarose embedded, irradiated genomic DNA as substrate [Cheong et al., 1998], CE under the conditions employed here does not support plasmid end joining (Fig. 1A). High DNA end joining activity directly detectable after staining of the gel is observed in reactions assembled with NE (Fig. 1A). Activity is extract concentration dependent and leads to the formation of dimers, trimers, and other higher order multimers. Formation of circles (relaxed or supercoiled) is not consistently detected under these conditions (see below). High level of activity is also observed in reactions assembled with WCE, although a slightly higher amount of extract is required to reach levels of activity equivalent to those of NE (Fig. 1A).

End joining is ATP dependent, and only limited dimer production is observed in reactions assembled without ATP (Fig. 1B); an optimum is reached at 1 mM, which is the

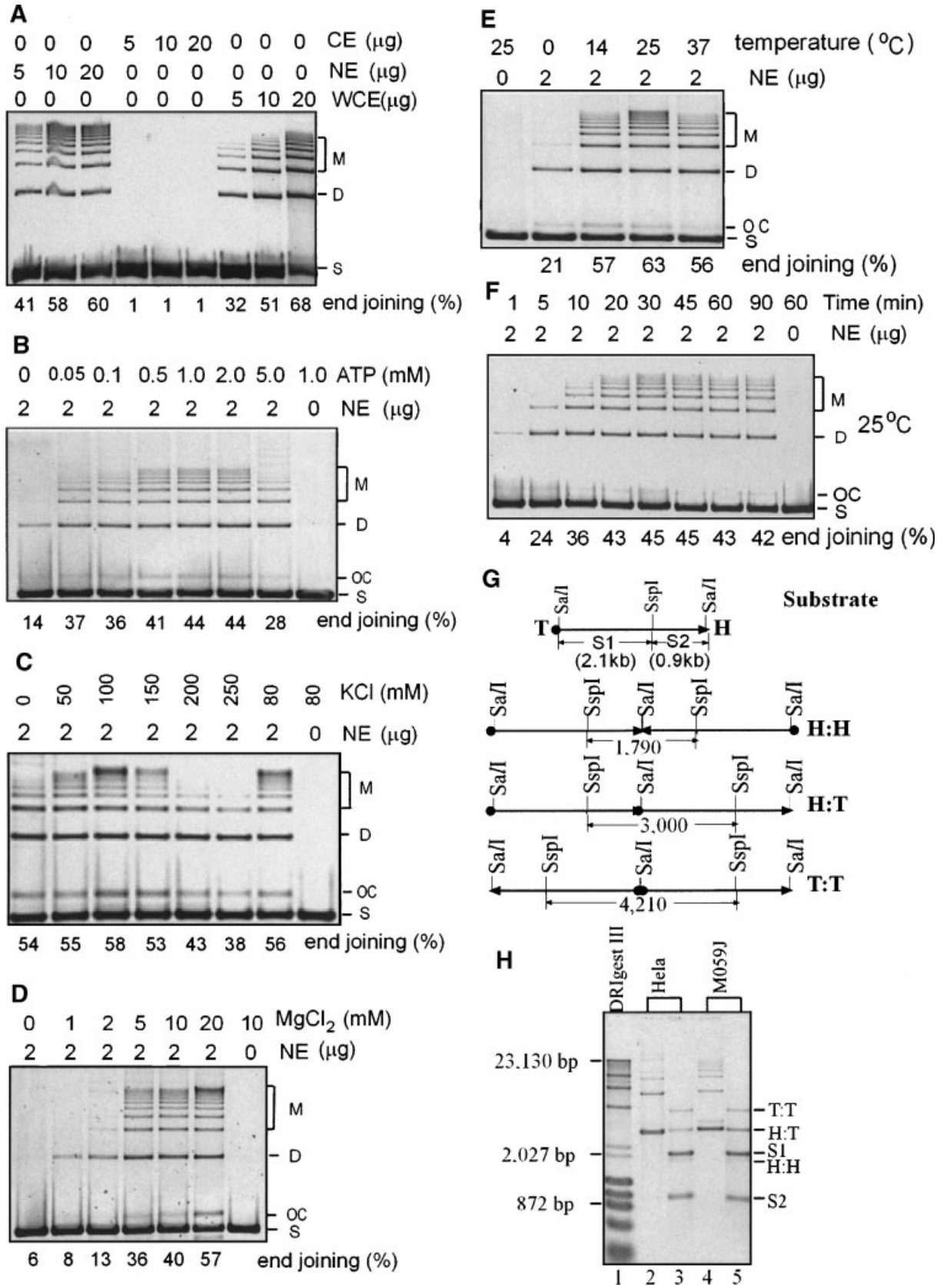

A
0 0 0 5 10 20 0 0 0 CE (μg)
5 10 20 0 0 0 0 0 0 NE (μg)
0 0 0 0 0 0 5 10 20 WCE(μg)
M
D
S
41 58 60 1 1 1 32 51 68 end joining (%)
B
0 0.05 0.1 0.5 1.0 2.0 5.0 1.0 ATP (mM)
2 2 2 2 2 2 2 0 NE (μg)
M
D
OC
S
14 37 36 41 44 44 28 end joining (%)
C
0 50 100 150 200 250 80 80 KCl (mM)
2 2 2 2 2 2 2 0 NE (μg)
M
D
OC
S
54 55 58 53 43 38 56 end joining (%)
D
0 1 2 5 10 20 10 MgCl2 (mM)
2 2 2 2 2 2 0 NE (μg)
M
D
OC
S
6 8 13 36 40 57 end joining (%)
E
25 0 14 25 37 temperature (°C)
0 2 2 2 2 NE (μg)
M
D
OC
S
21 57 63 56 end joining (%)
F
1 5 10 20 30 45 60 90 60 Time (min)
2 2 2 2 2 2 2 2 0 NE (μg)
M
D
25°C
OC
S
4 24 36 43 45 45 43 42 end joining (%)
G
Substrate
SalI
SspI
SalI
T
H
S1
S2
(2.1kb) (0.9kb)
H:H
1,790
H:T
3,000
T:T
4,210
H
DRIgest III
Hela
M059J
23,130 bp
2,027 bp
872 bp
T:T
H:T
S1
H:H
S2
1 2 3 4 5

Fig. 1.

standard concentration employed here. KCl (Fig. 1C) is not required for end joining, but activity increases, particularly in the region of higher order multimers, in the presence of 50–150 mM KCl; higher concentrations inhibit end joining. Low level of circle formation is observed in these experiments that follows similar salt concentration dependence. Similar results are obtained with NaCl (not shown). Because the predominant monovalent metal cation intracellularly is K^+, it is selected as a component in the reaction buffer at 80 mM.

DNA end joining requires Mg^{2+} as activity is undetectable in reactions assembled without Mg^{2+} and very low below 5 mM (Fig. 1D). Mn^{2+} can substitute for Mg^{2+} but slightly higher concentrations are required to achieve equivalent levels of activity (not shown). Ca^{2+} up to 20 mM is unable to support in vitro DNA end joining (results not shown). On the basis of these results, Mg^{2+} is included in the reaction buffer as a bivalent cation at 10 mM, a concentration between the 2–4 mM present in interphase nucleus and the 4–17 mM present during M-phase [Strick et al., 2001].

End joining is temperature dependent, but activity is detected even in samples kept in ice (~0°C) for 1 h (Fig. 1E). Near optimum end joining is measured after 1 h at 14°C and a maximum is reached at 25°C. Activity is slightly reduced in reactions incubated at 37°C. End joining is fast and products are detected even after 1 min incubation at 25°C. At this temperature, end joining reaches a plateau at 20–30 min (Fig. 1F) with approximately 45% of the input substrate joined to dimers and multimers. Faster end joining is observed at 37°C with nearly 20% of substrate joined to a dimer in 1 min and a plateau reached in 10 min (not shown). Further experiments are carried out at 25°C for 1 h, as this temperature gives maximum end joining and limits nuclease activity occasionally present in the extracts.

The products detected in the above reactions can form by joining the input plasmid in different orientations as depicted in Figure 1G. If we define as head (H) the end of the substrate proximal to the *Ssp* I site, analysis of product following digestion with this restriction endonuclease reveals the preference of the reaction for head to head (H:H), head to tail (H:T), or tail to tail (T:T) end joining. Since random end joining would produce twice as many head-to-tail products than head-to-head or tail-to-tail, our results (Fig. 1H) are compatible with random end joining.

The above results provide an optimization and initial characterization of the end joining reactions that are used in the following sections to study the role of DNA-PK in DNA end joining in vitro.

Role of DNA-PK in DNA End Joining in Wild Type Cells

To study the role of DNA-PK in DNA end joining, we employed as a tool wortmannin, an

Fig. 1. Reaction standardization, kinetics, and products analysis during in vitro end joining. **A**: DNA end joining in reactions assembled with the indicated amount of cytoplasmic extract (CE), nuclear extract (NE), or whole cell extract (WCE) of HeLa cells. Reactions (20 μl) were assembled with the indicated amount of extract and 250 ng plasmid substrate and incubated at 25°C for 1 h. Other conditions are as described under ''Materials and Methods.'' Products were analyzed by electrophoresis in a 0.7% agarose gel run at 45 V (2 V/cm) for 5 h. Gels were stained in SYBR Gold and scanned in a FluorImager. Quantification was carried out using the ImageQuant software. The results were used to calculate percent plasmid migrating as a dimer and other higher order forms. This value is given at the bottom of the gel (% end joining). The migration distances of the input plasmid substrate (S), as well as of dimers (D), and other higher order forms (M) are indicated. In some of the reactions, open circles (OC) are formed. **B**: Effect of ATP on DNA end joining. Two micrograms of HeLa NE was used in all reactions with the standard reaction buffer adjusted to the indicated amounts of ATP. **C**: Effect of KCl on DNA end joining; other components are at the concentrations given for the standard reaction buffer. **D**: Effect of $MgCl_2$ on DNA end joining. Other details as in A. **E**: DNA end joining was carried out at the indicated temperatures for 1 h. Reactions were assembled with 2 μg HeLa NE under the conditions described under ''Materials and Methods.'' **F**: Kinetics of DNA end joining in reactions incubated at 25°C. Other details as in A. **G**: Possible arrangements of DNA ends in the end joining reaction. The end close to the *Ssp* I site of the substrate is defined as head (H), the one remote from this site as tail (T). Intermolecular joining can lead to junctions combining two heads (H:H), two tails (T:T), or a head and a tail (H:T). The different end joining configurations can be distinguished by digestion with *Ssp* I, which will produce the indicated diagnostic fragments in rejoined products and fragments S1 and S2 in the products or in the substrate that has not undergone end joining. **H**: Restriction analysis of DNA end joining products in reactions assembled with HeLa, or M059J WCE. Reactions assembled with 20 μg WCE were incubated at 25°C for 1 h and DNA was purified. One half of this DNA was digested by *Ssp* I. Digested and non digested DNA was run on a 0.7% agarose gel, along with a DRIgest III as DNA molecular weight marker. Shown are the sizes of resulting fragments diagnostic for the different forms of DNA end joining, as well as the sizes of non diagnostic fragments generated by the digestion of the input substrate and, partly, of the products (S1 and S2).

irreversible inhibitor of the kinase. Wortmannin was originally characterized as a PI-3 kinase inhibitor, but higher concentrations were later shown to also inhibit other kinases of the PI-3 related family of protein kinases, such as DNA-PK, ATM, and ATR [Powis et al., 1994; Sarkaria et al., 1998]. Wortmannin effectively inhibits in vitro DNA end joining in reactions assembled with WCE, but the effect is strongly dependent on the timing of addition of wortmannin in relation to other components of the reaction buffer. Wortmannin (2 μM) added just before transferring assembled reactions to 25°C leaves DNA end joining unaffected (results not shown), indicating that pre-incubation is required. When the otherwise complete reaction is pre-incubated for 10 min with 2 μM wortmannin at 25°C and then DNA and ATP are added before a further 1 h incubation for end joining, nearly complete inhibition is observed compared to control reactions assembled with DMSO, the solvent of wortmannin (Fig. 2A). A similar level of inhibition is observed when DNA is included with wortmannin in the pre-incubation reaction mixture. However, when ATP is included with wortmannin in the pre-incubation reaction mixture an abrogation of the inhibitory effect is observed, as ATP competes with wortmannin for binding to the active site of DNA-PKcs thus reducing its inhibitory effect [Sarkaria et al., 1998]. On the basis of these observations, reactions are pre-treated for 10 min with wortmannin in the absence of ATP. To prevent DNA end joining under the suboptimal pre-incubation conditions, substrate DNA was also omitted from the pre-incubation reaction mixture.

The effect of wortmannin on reactions assembled with WCE is concentration dependent with low levels of inhibition observed at 0.1 μM, half maximum inhibition between 0.2 and 0.5 μM and maximum inhibition between 0.5 and 1 μM (Fig. 2B). Inhibition of end joining by wortmannin closely parallels inhibition of DNA-PK activity assayed independently as described under "Materials and Methods," pointing to a correlation between the two effects (Fig. 2B; lower panel). Surprisingly, inhibition of DNA end joining by wortmannin is significantly reduced in reactions assembled with NE (Fig. 2C), despite the fact that the drug inhibits DNA-PK activity with similar efficiency and to similar final levels as in WCE (Fig. 2C; lower panel). Since comparable levels of DNA-PK activity appear to be detected in NE and WCE (Fig. 2B,C), we initially hypothesized that the stronger inhibition in WCE extract derives from factors absent in the NE, probably due to their fractionation in the CE. We inquired therefore whether wortmannin induced inhibition of DNA end joining can be restored in NE through the addition of CE. Indeed, reactions assembled with a mixture of NE and CE of HeLa cells show a stronger inhibition of end joining after treatment with wortmannin than reactions assembled with NE

Fig. 2. Effect of wortmannin on DNA end joining in HeLa cell extracts. **A**: Reactions were assembled with 20 μg of HeLa WCE but without ATP or DNA as indicated and pre-incubated with 2 μM wortmannin for 10 min at 25°C. After this pre-incubation period remaining components were added and end joining was allowed to take place at 25°C for 1 h. Pre-incubation with DMSO, the solvent of wortmannin has no effect on DNA end joining (**lane 1**). Nearly complete inhibition is observed when wortmannin is pre-incubated in the absence of ATP and DNA (**lane 2**). Pre-incubation of wortmannin in the presence of ATP completely abolishes the inhibitory effect of wortmannin (**lane 3**). Pre-incubation of wortmannin with DNA preserves the inhibitory effect of wortmannin on DNA end joining (**lane 4**). **B**: Reactions were assembled with 20 μg HeLa WCE and pre-incubated with the indicated concentrations of wortmannin for 10 min before adding DNA and ATP to initiate end joining. Five microliters of treated reaction mixture was used to also assay DNA-PK activity as described under ''Materials and Methods.'' The **upper panel** shows DNA end joining, while the **lower panel** DNA-PK activity. **C**: Wortmannin is less effective when added to reactions assembled with HeLa NE. Ten micrograms of HeLa NE extract was pre-incubated with 0, 0.1, 0.2, 0.5, 1, 2, 5 μM wortmannin in 17 μl of NHEJ reaction buffer without DNA and ATP at 25°C for 10 min, then DNA and ATP were added to start the reaction, or 5 μl of treated reaction mixture was used to measure DNA-PK activity. The upper figure shows DNA end joining, while the lower figure DNA-PK activity. **D**: Effect of HeLa and M059J CE on wortmannin induced inhibition of DNA end joining in reactions assembled with 2 μg HeLa NE. HeLa NE was combined with 1 or 2 μl of HeLa CE (7.7 μg/μl), or M059J CE (8 μg/μl), then treated with 2 μM wortmannin before initiating DNA end joining as described above. **E**: Wortmannin inhibition depends on the amount of HeLa NE added in the reaction. 2, 4, and 6 μg of HeLa NE were treated with 2 μM wortmannin, or were mock treated, and then tested for end joining as described above. **F**: Wortmannin inhibition of end joining can be enhanced by purified DNA-PK. 2 μg of HeLa NE was combined with 1.6 μg purified DNA-PK, treated with 2 μM wortmannin, or left untreated, and DNA end joining was measured as described above. **G**: The effect of wortmannin on DNA end joining depends on the amount of substrate DNA used to assemble reactions. Two micrograms of HeLa NE were treated with 2 μM wortmannin as described above, or were mock treated, and then substrate DNA at the indicated amounts was given plus ATP to initiate end joining.

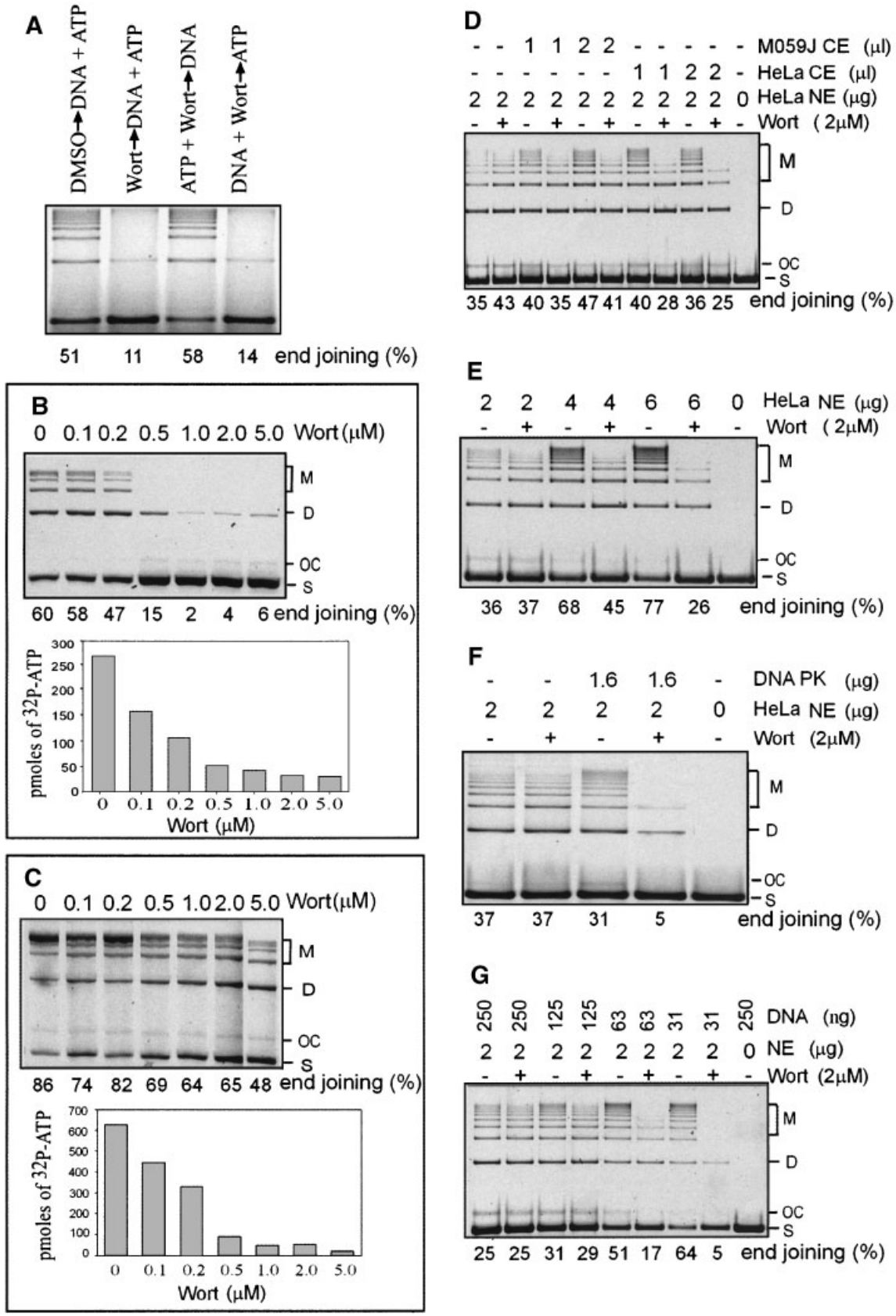

A
DMSO→DNA + ATP
Wort→DNA + ATP
ATP + Wort→DNA
DNA + Wort→ATP
51 11 58 14 end joining (%)
B
0 0.1 0.2 0.5 1.0 2.0 5.0 Wort (μM)
M
D
OC
S
60 58 47 15 2 4 6 end joining (%)
pmoles of 32P-ATP
Wort (μM)
C
0 0.1 0.2 0.5 1.0 2.0 5.0 Wort(μM)
86 74 82 69 64 65 48 end joining (%)
pmoles of 32P-ATP
Wort (μM)
D
M059J CE (μl)
HeLa CE (μl)
HeLa NE (μg)
Wort (2μM)
35 43 40 35 47 41 40 28 36 25 end joining (%)
E
HeLa NE (μg)
Wort (2μM)
36 37 68 45 77 26 end joining (%)
F
DNA PK (μg)
HeLa NE (μg)
Wort (2μM)
37 37 31 5 end joining (%)
G
DNA (ng)
NE (μg)
Wort (2μM)
25 25 31 29 51 17 64 5 end joining (%)

Fig. 2.

alone (Fig. 2D). This increase may be either due to an unknown factor present in the CE, as initially hypothesized, or to DNA-PK itself, as CE contains DNA-PK at levels comparable to those of NE (results not shown). To distinguish between the two possibilities, we assembled reactions in which NE of HeLa cells was mixed with CE from M059J cells. These cells lack DNA-PKcs (see below) but should contain the additional factors presumed to enhance the effect of wortmannin on DNA end joining. If a factor partitioning in the CE were responsible for the enhanced inhibition by wortmannin, it should exert activity in the M059J cell extract as well. If, on the other hand, the effect is due to DNA-PK, no additional effect would be expected. CE of M059J cells added to reactions assembled with NE of HeLa cells has only a small effect (Fig. 2D), pointing to DNA-PK as a key parameter determining the level of wortmannin induced inhibition of DNA end joining. This interpretation is further supported by the increased wortmannin induced inhibition of DNA end joining in reactions assembled with progressively higher amounts of NE (Fig. 2E). Under these conditions, DNA end joining is progressively enhanced, as is also the inhibition achieved after treatment with wortmannin. As a final test, we examined the effect of purified DNA-PK on the level of wortmannin induced inhibition of DNA end joining. The results (Fig. 2F) demonstrate a strong enhancement of wortmannin induced inhibition of DNA end joining after addition of 1.6 μg purified DNA-PK in reactions assembled with NE.

DNA-PK binds DNA ends and when inhibited by wortmannin is thought to remain bound, blocking thus assess to other proteins [Calsou et al., 1999]. This behavior explains the observed inhibition of DNA end joining and suggests that the stoichiometry between DNA ends and DNA-PK molecules is critical for the level of inhibition achieved. If more ends than DNA-PK molecules are present in the reaction, a significant number will remain free of DNA-PK and available to be rejoined by mechanisms operating in the absence of DNA-PK. To test the validity of this interpretation from a different view point we assembled reactions in which the amount of substrate DNA was progressively reduced while the amount of NE and thus the total amount of DNA-PK present in the reaction was kept constant. The results (Fig. 2G) show that reduction in the amount of DNA not only causes an increase in the fraction of substrate undergoing end joining, but also a drastic increase in wortmannin induced inhibition.

Active End Joining in Extracts of DNA-PKcs Deficient Cells

The results presented above suggest that DNA-PK, when present at sufficiently high concentrations, has the ability to bind available ends with the ultimate goal of shunting them into a DNA-PK dependent pathway of end joining (D-NHEJ). However, the same results are also suggesting the operation of a wortmannin resistant and therefore DNA-PK independent pathway of end joining when DNA-PK is present at subsaturating concentrations and ends remain free of DNA-PK. For a more conclusive evaluation of the role of DNA-PK in DNA end joining and the possible operation of DNA-PK independent pathways, we carried out experiments using extracts of M059J cells. These cells have a frameshift mutation in the gene encoding DNA-PKcs leading to the production of unstable mRNA. As a result, M059J cells are practically DNA-PK null, radiosensitive to killing by IR and other DNA DSB inducing agents, and defective in DNA DSB rejoining [Allalunis-Turner et al., 1993; Lees-Miller et al., 1995; Anderson et al., 2001].

WCE prepared from M059J cells support active DNA end joining in an extract concentration dependent manner (Fig. 3A) confirming that DNA-PK is not required for DNA end joining. Wortmannin has no effect on DNA end joining in reactions assembled with WCE of M059J cells, as expected from the absence of DNA-PKcs, but also indicating that inhibition of other kinases of the PI3K family has no effect on DNA end joining (Fig. 3B). Addition of purified DNA-PK in these reactions causes a modest increase in DNA end joining and renders them sensitive to inhibition by wortmannin (Fig. 3B). There is no detectable DNA-PK activity in M059J cells, and similar to observations with HeLa cells, wortmannin causes a complete inhibition of the added kinase activity (Fig. 3B, lower panel). Because M059J cells are deficient only in the kinase component of DNA-PK, but have normal levels of Ku, we examined whether addition of only the purified catalytic subunit is able to restore sensitivity to wortmannin. It is evident from the results in Figure 3C that DNA-PKcs restores sensitivity of the end joining reaction to wortmannin. This is

accompanied by a recovery in DNA-PK activity (Fig. 4C, lower panel), that is practically completely inhibited by wortmannin.

End joining in reactions assembled with extracts of M059J cells appears random with no clear indication for a preference for H:H or T:T end joining (Fig. 1G,H). Similar to results with HeLa cells, end joining in extracts of M059J cells is mostly error-free, as over 90% of the products can be re-digested by *Sal* I, the restriction endonuclease used to linearize the plasmid substrate (Fig. 3D). Finally, similar trends are observed, for the most part, in the joining of ends produced by different restriction endonucleases. Thus, ends produced by *Sal* I, *Bam* HI, and *Sma* I are rejoined with similar efficiency in extracts of HeLa and M059J cells, although in both cell lines blunt ends are rejoined with significantly lower efficiency than ends with 5′ protruding single strands (Fig. 3E). Interestingly, ends with 3′ protruding single strands are rejoined with significantly higher efficiency in M059J cells (Fig. 3E). The possible significance of this phenomenon which appears to be further enhanced by depletion of Ku has been discussed [Wang et al., 2003]. Together, the above results indicate similar overall properties of end joining in the presence or absence of DNA-PK, and suggest the function of alternative pathways of DNA end joining with operational manifestations similar to those of D-NHEJ under the in vitro conditions employed here.

DISCUSSION

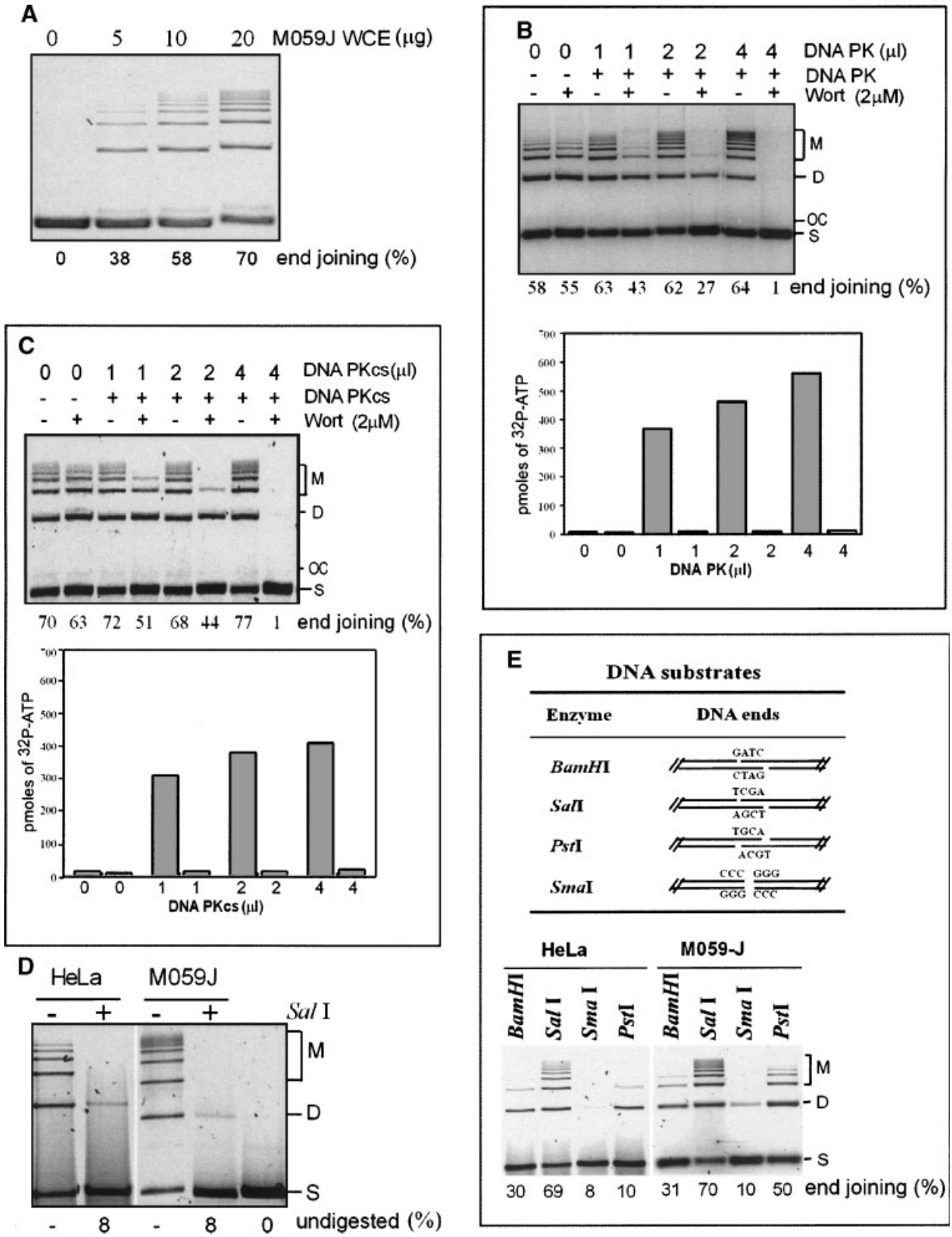
Genetic studies quantitatively evaluating the kinetics of rejoining of radiation-induced DNA DSBs in wild type cells of higher eukaryotes [Dikomey and Franzke, 1986; Metzger and Iliakis, 1991], in mutants deficient in DNA-PKcs [Nevaldine et al., 1997; DiBiase et al., 2000a], Ku [Wang et al., 2001b], or DNA ligase IV [Wang et al., 2001c], as well as after treatment with wortmannin [Wang et al., 2001a,b,c] provide evidence for the operation of at least two pathways of NHEJ with distinct properties that we have termed D-NHEJ and B-NHEJ [Wang et al., 2001b, 2003] (see also "Introduction"). D-NHEJ is fast ($t_{1/2}$: 5–30 min), requires DNA-PKcs, Ku, DNA ligase IV, and probably Xrcc4 and rejoins DNA ends without excessive degradation [Wang et al., 2001b, 2003]. B-NHEJ on the other hand is slow ($t_{1/2}$ 2–20 h), may involve degradation of DNA ends [Loebrich et al., 1995], and utilizes as of yet unidentified components [Wang et al., 2001b, 2003]. The two pathways operate in a complementary fashion and cooperate to remove IR-induced DNA DSBs in a well-defined hierarchical order. D-NHEJ is dominant and normally removes the majority of DNA DSBs. When D-NHEJ is chemically or genetically compromised, B-NHEJ acts as a backup to remove, practically completely, DNA DSBs, albeit with slower kinetics [DiBiase et al., 2000b]. The biochemical experiments presented in the previous section extend and complement the genetic studies by reproducing several important, albeit not all, of the features of these pathways.

How D-NHEJ Dominates DNA End Joining

Inhibition of in vitro DNA end joining in reactions treated with wortmannin is compatible with a blocking of the DNA substrate ends by the inactive kinase [Calsou et al., 1999]. The complete inhibition observed in reactions treated with excess DNA-PK suggests that the interaction between DNA ends and DNA-PK has a considerable kinetic advantage over several other possible interactions with activities present in the extract. This kinetic advantage will be exploited in the cell to shunt captured ends to a DNA-PK dependent pathway of end joining. Essential for the shunting of DNA ends to the DNA-PK dependent pathway of end joining is the presence of adequate amounts of DNA-PK. The results in Figure 2 suggest that there is stoichiometric requirement for DNA-PK and DNA ends for the inhibition to be complete.

In the in vitro reaction the level of inhibition is determined by the proportion of ends that are captured by DNA-PK (Fig. 4). In vivo where diffusion of molecules the size of DNA-PKcs, or of DNA ends, in view of the chromatin organization of the DNA, is expected to be restricted, the presence of DNA-PKcs in the vicinity of the break (i.e., the induction of the break within a DNA-PK surveillance domain [DiBiase et al., 2000a]) will be required for the shunting of the break in a DNA-PK independent pathway of end joining (Fig. 4).

The avid binding of DNA-PK on DNA ends explains how D-NHEJ dominates NHEJ in-vivo. The results presented here imply that DNA ends generated either endogenously, or by


A
0 5 10 20 M059J WCE (μg)
0 38 58 70 end joining (%)
B
0 0 1 1 2 2 4 4 DNA PK (μl)
- - + + + + + + DNA PK
- + - + - + - + Wort (2μM)
M
D
OC
S
58 55 63 43 62 27 64 1 end joining (%)
pmoles of 32P-ATP
0 0 1 1 2 2 4 4
DNA PK (μl)
C
0 0 1 1 2 2 4 4 DNA PKcs(μl)
- - + + + + + + DNA PKcs
- + - + - + - + Wort (2μM)
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D
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70 63 72 51 68 44 77 1 end joining (%)
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0 0 1 1 2 2 4 4
DNA PKcs (μl)
D
HeLa M059J
- + - +
Sal I
M
D
S
- 8 - 8 0 undigested (%)
E
DNA substrates
Enzyme DNA ends
BamHI GATC CTAG
SalI TCGA AGCT
PstI TGCA ACGT
SmaI CCC GGG GGG CCC
HeLa M059-J
BamHI Sal I Sma I PstI BamHI Sal I Sma I PstI
M
D
S
30 69 8 10 31 70 10 50 end joining (%)

Fig. 3.

exogenous agents such as IR and other DNA damaging agents, will be quickly bound to DNA-PK and processed by D-NHEJ. We have previously presented a model to accommodate these observations [DiBiase et al., 2000a; Wang et al., 2003]. The model assumes that in the presence of Ku and DNA-PKcs, DNA ends are quickly captured and chromatin structure is locally altered to facilitate synapsis. This is considered as the rate-limiting step of the reaction and is thought to occur close to the nuclear matrix mediated by a complex set of protein-protein interactions between DNA-PKcs, Ku, DNA ligase IV, XRCC4, histones, and other unidentified factors. End joining is mainly catalyzed by DNA ligase IV. The high speed of the process and local changes in chromatin structure facilitate joining of correct ends and suppress the formation of chromosome aberrations. In the absence of Ku or DNA-PKcs, DNA ends are not captured and local changes in chromatin structure fail to occur. Ends remain open and are processed by components of B-NHEJ with slow kinetics (hours) because of inefficient synapsis at random locations in the nucleus. The long-lived DNA ends may interact with other DNA ends in the vicinity, generated by IR or other processes, causing the formation of chromosome aberrations.

After treatment with wortmannin, DNA-PK remains bound to the ends of the substrate DNA [Calsou et al., 1999] unable to facilitate remaining steps of D-NHEJ and blocking the inception of alternative mechanisms of end joining. In vitro, this blockade cannot be reversed by prolonged incubation of the reaction mixture (results not shown), and may be irreversible. Because in intact cells inhibition of DNA-PK by wortmannin generates a phenotype practically indistinguishable from that of cells lacking DNA-PKcs, or DNA ligase IV [DiBiase et al., 2000b; Wang et al., 2001c], it is possible that the inhibited kinase is either prevented from interacting with the DNA ends generated by the subsequently following radiation, or are freed up from the kinase by an unknown mechanism allowing the operation of alternative pathways of end joining (see below). Clearing, or a freeing-up, will be facilitated by the prolonged (5–20 h) half times of the DNA-PK independent pathways of end joining.

Alternative Pathways of NHEJ

When the number of DNA ends exceeds the number of DNA-PK molecules in the reaction, DNA ends are expected to remain free and to be processed for end joining by DNA-PK independent mechanisms (Fig. 4). These alternative pathways operate in extracts of wild type cells under conditions of low DNA-PK availability as compared to the number of DNA ends available, and their operation is indicated by the partial resistance of the reaction to wortmannin. The active end joining observed in such reactions indicates availability in sufficient amounts of the activities required for this processing. This assumption is further supported by the observation that extracts of M059J cells that lack DNA-PKcs are able to support active end joining. Thus, the absence of a key component of D-NHEJ is not compromising end joining in line with the operation of an alternative, DNA-PK-independent pathway that may be operationally equivalent to the genetically defined B-NHEJ [Asaad et al., 2000; DiBiase et al., 2000a,b; Wang et al., 2001b,c].

Fig. 3. DNA end joining in extracts of M059J cells. **A**: DNA end joining in M059J WCE. Reactions were assembled with the indicated amount of extract, and end joining measured under standard conditions. **B**: Effect of wortmannin on DNA end joining in reactions assembled with M059J WCE (20 μg) in the presence or absence of purified DNA-PK (0.8 μg/μl). The reaction mixture was pre-treated as described in Figure 2 and subsequently one part used to assemble the end joining reaction by adding DNA and ATP (**upper panel**), while the rest was used to measure DNA-PK activity (**lower panel**). **C**: Effect of wortmannin on DNA end joining in reactions assembled with M059J WCE (20 μg) in the presence or absence of purified DNA-PKcs (0.2 μg/μl). The reaction mixture was pre-treated as described in Figure 2 and subsequently one part used to assemble the end joining reaction by adding DNA and ATP (upper panel), while the rest was used to measure DNA-PK activity (lower panel) **D**: Fidelity of DNA end joining, as measured by *Sal* I digestion of purified DNA, from reactions assembled with HeLa or M059J WCE. Half of the purified products were digested with *Sal* I (+) and fractionated on a 0.7% agarose gel along with the undigested portion of the reaction. Percent-undigested plasmid was calculated by Image-Quant and the values obtained are shown at the bottom of the gel. Note that the products of the end joining reaction remain sensitive to *Sal* I under the conditions examined (92% digestibility) suggesting joining without end modification. The results of HeLa and M059J cells are from different gels, therefore the bands do not align. **E**: End joining of substrate generated from pSP65 by digestion with four different restriction endonucleases (*Bam H*I, *Sal* I, *Pst* I, and *Sma* I) to generate the types of ends shown in the upper panel. The lower panel shows DNA end joining under standard conditions in reactions assembled with extracts of HeLa or M059J cells with the substrates shown in the upper panel. Percent end joining is shown at the bottom of the gel.

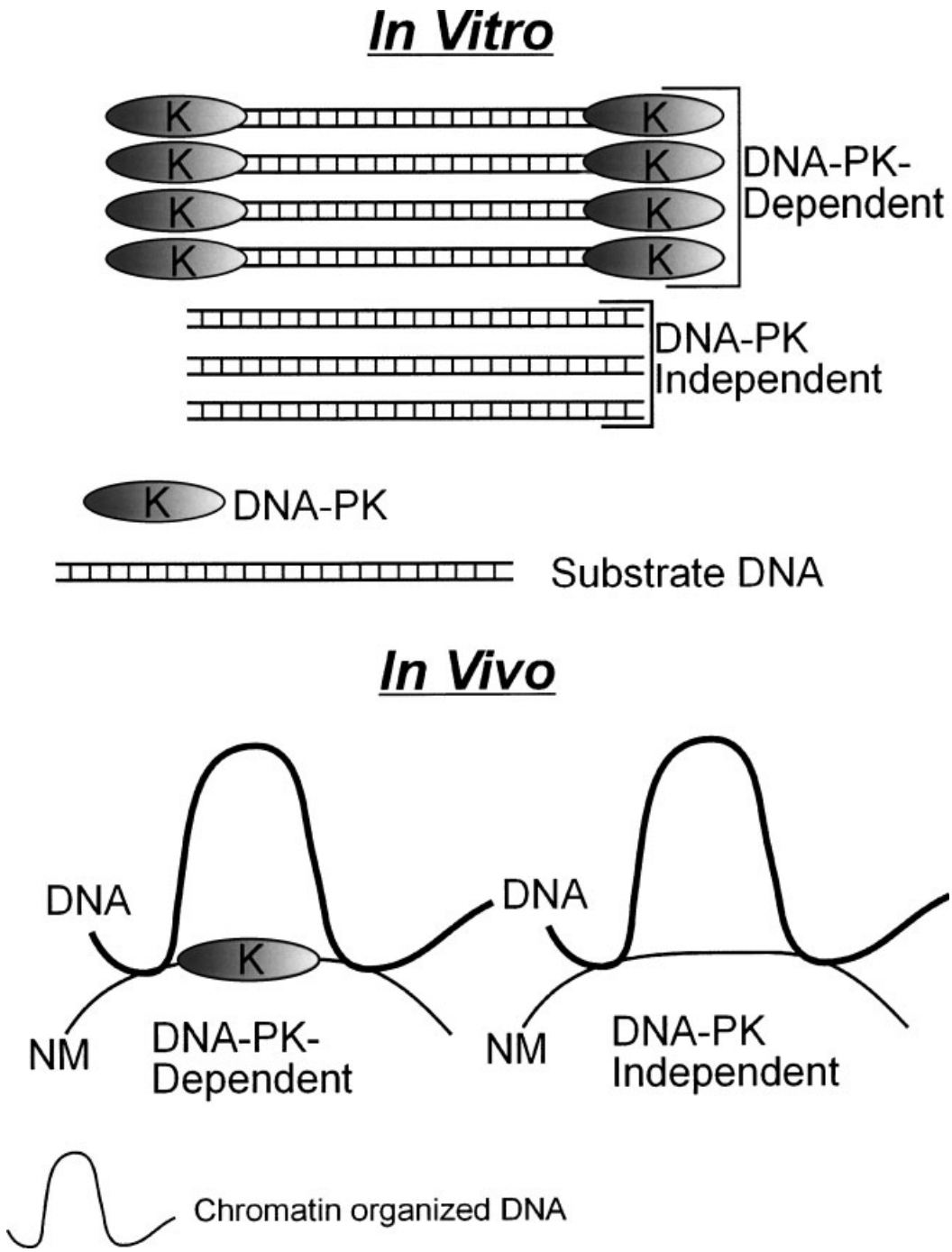

Fig. 4. Model for DNA-PK dependent and independent end joining in vivo and in vitro. In vitro, a fraction of substrate ends, depending on DNA-PK availability, is captured and shunt into a DNA-PK dependent pathway of end joining. This form of end joining is sensitive to inhibition by wortmannin. Remaining ends are captured and rejoined by activities operating in the absence of DNA-PK, which renders this form of end joining wortmannin resistant. In vivo, DSBs induced in regions with proximity to DNA-PK (DNA-PK surveillance domains) are rejoined fast by a DNA-PK dependent pathway of end joining. DSBs induced outside a DNA-PK surveillance domain, or in cells lacking DNA-PK, are rejoined with much slower kinetics by a DNA-PK independent pathway.

This hypothesis is in agreement with earlier reports that end joining of transfected DNA remains at levels similar to those of wild type controls in cells deficient in DNA-PKcs [Harrington et al., 1992; Chang et al., 1993; Verkaik et al., 2002], Ku [Kabotyanski et al., 1998; Verkaik et al., 2002], XRCC4 [Kabotyanski et al., 1998; Verkaik et al.,

2002], or DNA ligase IV [Verkaik et al., 2002] pointing to pathways operating efficiently in the absence of these proteins. Sequencing of junctions generated in cells with such defects suggested a shift from homology independent to microhomology dependent end joining [Kabotyanski et al., 1998; Verkaik et al., 2002]. Therefore, frequently this form of end joining is also referred to as microhomology dependent end joining. Similar results were also obtained in vitro and implicated Ku in the fidelity of end joining, as well as in the inhibition of the pathway utilizing microhomologies [Labhart, 1999; Feldmann et al., 2000; Chen et al., 2001; Verkaik et al., 2002]. Alternative pathways of DNA DSB end joining have also been suggested from studies involving the generation of knock-out DT40 cells for DNA ligase IV and Ku70 [Adachi et al., 2001]. Thus the evidence for alternative pathways of NHEJ is ample and quite diverse. There are distinct parallels between microhomology dependent end joining and B-NHEJ that require further study. These studies will benefit substantially by a biochemical characterization of B-NHEJ.

Kinetics and Fidelity of B-NHEJ In Vivo and In Vitro

The kinetic advantage of D-NHEJ in vivo (see "Introduction" and above) is not reproduced in vitro, where both D-NHEJ and B-NHEJ operate with similar kinetics. We attribute this difference to the loss of the kinetic advantage in the synapsis step of D-NHEJ, and have discussed it before [Wang et al., 2003], as synapsis may not be rate limiting at the high DNA concentrations used in vitro.

The in vitro assay utilized in the present study, as well as in previous reports [Gu et al., 1996; Baumann and West, 1998; Labhart, 1999], fails to reproduce the reduced fidelity of B-NHEJ in vivo (see "Introduction") and the cancer prone nature of alternative pathways of NHEJ in mice [Difilippantonio et al., 2000; Gao et al., 2000; Zhu et al., 2002]. We propose that this may also be a reflection of the artificial kinetic advantage of B-NHEJ under the in vitro conditions employed here. Indeed, a fidelity reduction has been reported in the absence of Ku under conditions supporting end joining less efficiently than in our experiments [Feldmann et al., 2000; Chen et al., 2001]. It is possible that even in vitro, slow end joining allows processing of the ends by an exonuclease (likely a 3′ to 5′ exonuclease) causing a reduction in fidelity and that this processing is modulated by Ku. That reduced fidelity in the absence of Ku may be seen in in vitro systems only under conditions of kinetic disadvantage is also supported by the high fidelity observed in experiments evaluating end joining of transfected plasmid DNA in Ku deficient cells [Kabotyanski et al., 1998].

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