Accurate in Vitro End Joining of a DNA Double Strand Break with Partially Cohesive 3’-Overhangs and 3’-Phosphoglycolate Termini

EFFECT OF Ku ON REPAIR FIDELITY

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To examine determinants of fidelity in DNA end joining, a substrate containing a model of a staggered free radical-mediated double-strand break, with cohesive phosphoglycolate-terminated 3’-overhangs and a one-base gap in each strand, was constructed. In extracts of Xenopus eggs, human lymphoblastoid cells, hamster CHO-K1 cells, and a Chinese hamster ovary (CHO) derivative lacking the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), the predominant end joining product was that corresponding to accurate restoration of the original sequence. In extracts of the Ku-deficient CHO derivative xrs6, a shorter product, consistent with 3’ → 5’ resection before ligation, was formed. Similar results were seen for a substrate with 5’-overhangs and recessed 3’-phosphoglycolate ends. Supplementation of the xrs6 extracts with purified Ku restored accurate end joining. In Xenopus and human extracts, but not in hamster extracts, gap filling and ligation were blocked by wortmannin, consistent with a requirement for DNA-PKcs activity. The results suggest a Ku-dependent pathway, regulated by DNA-PKcs, that can accurately restore the original DNA sequence at sites of free radical-mediated double-strand breaks, by protecting DNA termini from degradation and maintaining the alignment of short partial complementarities during gap filling and ligation.

Cells deficient in any of the three components of DNA-dependent protein kinase DNA-PK (i.e., the catalytic subunit DNA-PKcs or either subunit of the DNA end binding heterodimer Ku) are partially deficient in the rejoicing of double strand breaks (DSBs) (1–4). The precise roles of the individual subunits in the repair process are not known, but possible functions have been suggested based on their known biochemical properties. Ku has been proposed to align DNA ends and thus promote ligation (5–7), to protect the ends from degradation (8), and/or to unwind duplex DNA ends and thus expose microhomologies that could be used for splicing the ends together (1, 9). DNA-PKcs has been proposed to regulate accessibility of DNA ends to processing (10), perhaps by promoting its own dissociation following autophosphorylation (11) and/or by allowing translocation of Ku from the ends into the interior of the DNA (10, 12, 13).

Ionizing radiation is a major environmental source of DSBs (14–16). Radiation-induced DSBs are formed by fragmentation of deoxyribose, typically leaving in each strand a one-base gap with 5’-phosphate and either 3’-phosphate or 3’-phosphoglycolate (PG) termini (14, 15, 17). Such DSBs present to repair systems a more complex substrate than simple restriction enzyme cuts, potentially increasing the possibility of errors during rejoining. In this report, we describe how synthetic substrates containing mimics of radiation-induced breaks, with defined geometry and chemical structure (see Figs. 1 and 2), are processed in several in vitro end joining systems. The results suggest a Ku-dependent repair process that can accurately restore the original DNA sequence at sites of these complex DSBs, through the use of very short residual complementarities at the ends of the break.

EXPERIMENTAL PROCEDURES

Materials—Hamster CHO and derivative cell lines were from the European Collection of Cell Culture (Wiltshire, United Kingdom), except for XR-C1 cells (18), which were obtained from M. Z. Zdzienicka (Leiden University). Extracts of Xenopus eggs (19), Chinese hamster fibroblasts (20), and human GM00558B lymphoblastoid cells (ATCC) (21) were prepared according to procedures described previously, except that for Xenopus egg extracts, the extraction buffer was 30 mM Tris-HCl, pH 7.9, 90 mM KCl, 10 mM sodium-p-glycero phosphat, 2 mM EGTA, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 100 μM N-acetyl-DEVD-aldehyde, the latter peptide added as a caspase inhibitor (22).

A plasmid substrate containing a site-specific DNA double strand break with 2-base cohesive 5’-overhangs and recessed 3’-PG termini (Fig. 2C) was prepared by ligation of 3’-PG oligomers into larger 5’-overhangs formed by controlled digestion of plasmid pSV54 with the 3’→5’ exonuclease activity of T4 polynucleotide kinase, as described (10, 23). A similar construct, but bearing a PG-terminated -ACG 3’-overhang was similarly constructed by ligation of slightly longer oligomers (pGCCGACCGCGACG* and 5’-ACG-terminated PCGACCGCGCGACG*) into the same prepared vector (Fig. 1). The structure of the 3’-PG oligomers was verified by Fourier transform electrospray ionization mass spectrometry; all species detected could be ascribed to isotopes or salts of the oligomer or to the internal standard, with no detectable contaminants (24).

As described previously (23), the 5’-overhang construct was purged of any residual molecules having 3’-hydroxyl termini by treatment with...
Extracts of CHO-K1 Cells Accurately Rejoin DSBs with Terminally Modified 3′-Overhangs and 1-Base Gaps—Radiation-induced DNA DSBs are formed when multiple radicals, emanating from a single ionization track, attack and fragment closely opposed deoxyribose moieties in both strands (28). To examine the repair of such lesions by end joining, a model DSB was constructed (Fig. 1). Each end of the break had a PG-terminated 3-base 5′-overhang with the sequence -ACG*. Thus, this substrate mimics the break that would result from free radical-mediated cleavage at each T in the self-complementary sequence ACGR. Accurate repair of such a break (Fig. 2A) would require annealing of the CG sequences in each overhang, removal of PG, fill-in of the 1-base gap in each strand (presumably using the 3′-overhang from the other end of the break as a template), and finally ligation. In order that processing of the break could be followed, it was labeled with 32P on 14 bases from one of the 3′ termini.

This substrate was incubated in CHO-K1 whole-cell extracts and then cut with restriction enzymes on each end to release short fragments, which were then analyzed on sequencing gels. As shown in Fig. 3A, the CHO-K1 extracts were able to rejoin such breaks, and the predominant end-joined product was a 43-base fragment, corresponding to accurate rejoining by annealing, gap filling, and ligation, as described above (Fig. 2). Analysis of repair joints in individual plasmid clones recovered from the repair reaction confirmed the sequence of this repair joint as well as its predominance among repair products (Table I). In theory, such a product could only be formed from the head-to-tail joining of two ends, each of which had the -ACG* overhang. To test this requirement, parallel experiments were performed with a substrate having an -ACG* overhang ligated into the XhoI side of the substrate only (Fig. 2B). The opposite end of this plasmid would have a 10-base 5′-overhang, formed as an intermediate in the construction (see Fig. 1) (23). As predicted, this “one-sided” construct did not yield any 43-base repair product, but instead yielded a mixture of end-joined products with fragment lengths of 42, 41, 40, 39, 37, and 35 bases (Fig. 3B). The 39-base product would correspond to removal of the 3′-overhang and fill-in of the 10-base 5′-overhang, followed by direct ligation of the resulting blunt ends. The 40-, 41-, and 42-base products would correspond to retention of 1, 2, and 3 bases of the single 3′-overhang, plus all bases of the 5′-overhang (Fig. 2B), as was confirmed by sequencing cloning of repaired plasmid (Table I). The 37- and 35-base products would correspond to annealing of 2 and 4 bases, respectively, of the CCGG sequences on opposite sides of the break, presumably preceded by a 5- or 7-base 3′ resection of the end with a 3′-overhang (Fig. 2B). Since the 3′-overhang construct will necessarily contain some one-sided construct due to incomplete ligation of the PG oligomers into the vector, some or all of the inaccurate joins may be derived from these contaminants; thus, the proportion of accurate joins for the full 3′-overhang construct is probably even higher than is experimentally observed (as much as 70% in some experiments) and may approach 100%. The higher apparent incidence of inaccurate repair events indicated by the sequencing data (Table I) is expected, since these data will include repair products from plasmids into which only the unlabeled oligomer had been ligated, but such products will not be visible in the gel assays (Fig. 3).

As a further verification that the 43-base product was indeed the result of single-base gap filling, the reactions were performed with various combinations of dNTPs (Fig. 3A). As expected, only dTTP was essential for generation of the 43-base product, supporting the proposal that it was formed by gap filling opposite the template adenine in each -ACG overhang. The absence of dATP had little effect on the product distribution but, curiously, reduced overall end joining efficiency. The complete absence of any dNTPs appeared to accelerate 3′ resection in extracts from all of the cell lines. Replacement of dTTP with ddTTP promoted formation of a 16-base species consistent with the expected trapping of an intermediate in
FIG. 2. End joining substrates and repair products. A, the 3'-PG(4)-3'-overhang substrate mimics the DSB that would result from free radical-mediated deoxyribose fragmentation on a 3-base 3'-stagger. Accurate repair (43-base product) would require alignment-based fill-in prior to ligation. B, the one-sided 3'-overhang substrate was included as a control to assess the effect of incomplete ligation in construction of the 3'-overhang substrate. In theory, it cannot form any 43-base product, but it could form a 42-base product by fill-in of the 10-base 5'-overhang, abutting of the ends, and continuation of fill-in using the 3'-overhang as a template; 41-, 40-, and 39-base products would result from removal of 1, 2, and 3 bases, respectively, from the 3'-overhang prior to fill-in. C, the 5'-overhang substrate lacks the 1-base gaps but is otherwise similar to a 2-base-5'-staggered free radical-mediated DSB. All three substrates can, by various degrees of 3'-resection and/or fill-in, be converted to an intermediate with self-complementary CGCG 5'-overhangs, the alignment of which would result in the 35-base repair product.

which the one-base gap had been filled in with ddTTP, preventing the final ligation (Fig. 3E).

Thus, the presence of overhangs with a partial complementarity of as little as 2 bases was sufficient to permit accurate end joining with restoration of the original sequence, while lack of such complementarity led to multiple repair products corresponding to various degrees of 3'-resection.

**Accurate End Joining Requires Ku but Not DNA-PKcs—** Ku is a heterodimeric DNA-binding protein that has been implicated in DSB repair in vivo (1–4) and has been found to promote end-to-end DNA association (5, 7, 29) and to stimulate DSB joining by mammalian DNA ligases in vitro (29, 30). To assess the possible effects of Ku on the efficiency and accuracy of end joining, parallel experiments were carried out using extracts of the Ku-defective CHO derivative xrs6 (31, 32). As shown in Fig. 3A and Table I, there was no detectable formation of the accurately joined, 43-base repair product in xrs6 extracts. Instead, the predominant product was the 35-base product, consistent with 7-base 3'-resection in each strand and annealing of the CGCG microhomology (see Fig. 2). In addition, there appeared to be more extensive 3' processing in the xrs6 than in the CHO-K1 extract. For xrs6 extracts, no unprocessed 3'-PG ends remained, and more than half of the radiolabel had been lost entirely, presumably due to resection at least several bases into the duplex region of the DNA. Likewise, sequencing revealed formation, in the xrs6 extracts, of several repair joints with even larger deletions at the break site (Table I). Sequencing also confirmed the predominance of the product with apparent splicing at the CGCG microhomology as well as the absence of any accurate repair joints.

To assess whether the differences between CHO-K1 and xrs6 extracts were due to presence or absence of Ku or to some ancillary difference between the cell lines, extracts were also prepared from an xrs6 derivative cell line that had been stably transfected with a hamster Ku80 gene (33). Unlike xrs6 extracts, these xrs6-Ku80 extracts were competent to form the accurately joined 43-base repair product (Fig. 3A and Table I). Moreover, the addition of purified human Ku also restored accurate repair in the xrs6 extracts, promoting formation of the 43-base product while suppressing formation of the 35-base product (Fig. 3A). However, whereas the accuracy of end joining was restored to near that of CHO-K1 extracts, the efficiency was much lower, with only 0.2% joining in the Ku-supplemented xrs6 extracts versus 7% joining in CHO-K1. The reason for this lower efficiency is not known, but it could reflect differences in Ku phosphorylation state, differences between the human and hamster proteins, or secondary effects of Ku deficiency on other proteins in the cell. Nevertheless, taken together, the results strongly suggest a specific and apparently absolute requirement for Ku in the accurate end joining of 3'-staggered, free radical-mediated DSBs.

Similar experiments were performed with a substrate having a 5-base recessed 3'-PG ends and cohesive 2-base 5'-overhangs. With this substrate (which, unlike an actual free radical-mediated DSB, would not have a 1-base gap in each strand when the ends were annealed), the presence of Ku likewise suppressed 3' → 5' resection and promoted accurate end joining, although the requirement was not absolute (Fig. 3C). For CHO-K1 and xrs6-Ku80 extracts, 89 and 88% of the head-to-tail joins at 6 h incubation resulted in the accurate, 37-base product, consistent with annealing of the existing 2-base 5'-overhangs. The xrs6 extract yielded primarily the 35-base product (80%), consistent with a 2-base 3'-resection followed by annealing of the CGCG microhomology (see Fig. 2), but it did yield some 37-base product (20%). The addition of purified human Ku to the xrs6 extracts restored accurate end joining (85% 37-base product), although the extent of end joining was only about half that seen with the CHO-K1 extract. All of the extracts yielded only very small amounts of the 18-base (simple annealing) and 16-base (2-base resection) head-to-head joining products, suggesting that recircularization was prevented over intermolecular dimerization. Again, there was more end processing (PG removal, fill-in, and, at longer times, loss of radiolabel) in xrs6 than in CHO-K1 extracts. Thus, either Ku transfection or direct Ku addition restored accurate end joining of the 5'-overhang substrate to the xrs6 extracts.

To assess whether end joining required DNA-PKcs, effects of the DNA-PKcs inhibitor wortmannin were examined. Me3SO solvent (2%) alone reduced both the efficiency and the accuracy of end joining. Wortmannin, at a concentration sufficient to...
completely suppress DNA-PK activity (10, 27, 34), had no additional effect on formation of the 35- and 37-base end joining products and produced only a modest reduction (less than 2-fold) in the formation of the 43-base product (Fig. 3D). To assess whether DNA-PKcs might play an essential role independent of its kinase activity, experiments were also performed with extracts of the CHO derivative XR-C1, which lacks detectable DNA-PKcs protein (18). Unlike xrs6 extracts, these extracts produced significant amounts of the 43-base end joining product (Fig. 3E), confirming that DNA-PKcs is not essential for accurate end joining in this system.

End Joining in Extracts of Human Cells and Xenopus Eggs Shows Similar Specificity—In extracts of Xenopus eggs, 3'-overhang PG substrates would correspond to partial or complete loss of the 10-base overhang (see Fig. 2). Unlike the mammalian extracts, these extracts also yielded small quantities of 29–34-base products, which would correspond to partial or complete loss of the 10-base overhang (Fig. 2B).

In Xenopus egg extracts, formation of all end-joined products was efficiently blocked by 10 μM wortmannin (>20-fold reduction in end-joined products; Fig. 4A), a concentration sufficient to completely suppress DNA-PK activity (27). Because joining was less sensitive to Me2SO in Xenopus extracts than in the mammalian extracts (possibly due to the lower temperature), the wortmannin effect was unambiguous. In addition, wort-

FIG. 3. End joining in hamster cell extracts and the effect of Ku deficiency. A–C, the internally labeled (*) 3'-PG-terminated (a) 3'-overhang (A), one-sided 3'-overhang (B), and 5'-overhang (C) substrates described in the legend to Fig. 2 were incubated for 6 h (A and B) or for 1, 6, or 16 h (C) with extracts of CHO-K1, Ku-deficient xrs6, or Ku- transfected xrs6-Ku80 (xrs-Ku) cells and then cut near each end with XhoI and BstXI (see Fig. 2) for analysis of end processing and end joining. The 43- and 37-base products reflect accurate head-to-tail end joining (recircularization) of the 3'-overhang and 5'-overhang substrates, respectively; the analogous head-to-head end joining products would be 24 and 18 bases. In some cases, purified Ku protein (0.1 or 0.4 μg) was added as indicated, with incubation for 6 h. All reactions contained a 50 μM concentration of each dNTP, except as indicated. In A and B, the 15PG band corresponds to the initial 3'-PG-terminated substrate. PG removal yields the 15-mer, and 3' resection yields shorter species, with the 12-mer corresponding to a blunt end. In A, a portion of the gel is reproduced at 10× contrast to show weak bands, and above that, a replicate experiment is shown in which the gel was exposed to x-ray film to improve resolution of closely spaced bands. (The dTTP-depleted sample was lost from the first experiment.) In C, the 10PG band represents the unprocessed recessed 3'-PG terminus, and the 12-mer represents fill-in to a blunt end. D, effect of 10 μM wortmannin on end joining; conditions and substrates are the same as in A and C. E, the 3'-PG-terminated 3'-overhang substrate was incubated for 6 h in extracts of CHO-K1 cells or of DNA-PKcs-deficient XR-C1 cells and analyzed as in A. The size markers (M) are 5'-end-labeled oligomers of the indicated lengths and with the same sequence as the expected end joining products or processing intermediates. DMSO, Me2SO.
various ddNTPs were present, all samples contained 2% Me2SO ends of the break, when it can be determined unambiguously from the joint sequence. Bold letters show the CGCG micronomology. (position expected for a 3'-9') indicates the break in the initial substrate. Indicates a direct repeat that might have been used for splicing. This species could be an intermediate corresponding to fill-in of intermediates. The repair joint was AA

The repair joint was GGAA

One joint also had an inserted A, giving AAA

One joint also had an additional 4-bp deletion

Predicted length of the top strand of the fragment that would be produced by fill-in of intermediates.

The sequence is shown in terms of the top strand in Fig. 2. The vertical line indicates the transition between sequences derived from opposite ends of the break, when it can be determined unambiguously from the joint sequence. Bold letters show the CGCG micronomology.

A

B

One-sided

Heat Inact.

+ +

Heat Inact.

+ +

DMSO

+ +

Wortmannin

+ +

ddNTP

+ +

ddTTP

+ +

ddGTP+ddCTP

M

Fig. 4. End joining in Xenopus egg extracts. A, the various substrates were incubated in extracts at 13 °C for 6 h and then cut with XhoI and BstXI, B, same as A, except that wortmannin (10 μM) and various ddNTPs were present, all samples contained 2% Me2SO (DMSO), and some samples were cut with XhoI alone so that end processing intermediates and head-to-head joining products, but not head-to-tail recircularization products, were detected. See Fig. 2 and the legend to Fig. 3 for description of labeled products and processing intermediates.

Table I

| Joint sequence | Length | CHO-K1 | xr6-6-Ka60 | xr6 |
|---------------|--------|--------|-----------|-----|
|               | 3'-Overhang | One-sided | 3'-Overhang | One-sided | 3'-Overhang |
| AAGCGGACGTCGGCGGTCT | 43 | 10 | 5 | |
| AAGCGGACGTCGGCGGTCT | 42 | 4 | 6 | 1 | 2 |
| AAGCGGACGTCGGCGGTCT | 41 | 2 | 2 | 1 | |
| AAGCGGACGTCGGCGGTCT | 41 | 1 | | | |
| AAGCGGACGTCGGCGGTCT | 40 | 4 | 4 | 1 | |
| AAGCGGACGTCGGCGGTCT | 39 | 4 | 1 | 1 | |
| AAGCGGACGTCGGCGGTCT | 38 | 1 | | | |
| AAGCGGACGTCGGCGGTCT | 37 | 3 | 3 | 4 | 1 |
| AAGCGGACGTCGGCGGTCT | 35 | 5 | 8d | 1 | 3 | 15 |
| Shorter | 1' | 1' | 1' | 3b | | |
| Total | 36 | 25 | 13 | 12 | 20 |

The sequence is shown in terms of the top strand in Fig. 2. The vertical line indicates the transition between sequences derived from opposite ends of the break, when it can be determined unambiguously from the joint sequence. Bold letters show the CGCG micronomology.

A

B

One-sided

Heat Inact.

+ +

Heat Inact.

+ +

DMSO

+ +

Wortmannin

+ +

ddNTP

+ +

ddTTP

+ +

ddGTP+ddCTP

M

Fig. 4. End joining in Xenopus egg extracts. A, the various substrates were incubated in extracts at 13 °C for 6 h and then cut with XhoI and BstXI, B, same as A, except that wortmannin (10 μM) and various ddNTPs were present, all samples contained 2% Me2SO (DMSO), and some samples were cut with XhoI alone so that end processing intermediates and head-to-head joining products, but not head-to-tail recircularization products, were detected. See Fig. 2 and the legend to Fig. 3 for description of labeled products and processing intermediates.

mannin prevented the formation of a species migrating in the position expected for a 3'-hydroxyl 16-mer (Fig. 4, A and B). This species could be an intermediate corresponding to fill-in of the expected 1-base gap in the labeled strand of the annealed ends, prior to ligation. However, as noted above, a 16-base resection-dependent head-to-head joining product could also be formed. To distinguish between these possibilities, ddNTPs were added in an attempt to trap the extension product prior to the final ligation step (Fig. 4B). The addition of either ddTTP or all four ddNTPs largely prevented formation of the 43-base end joining product and increased the intensity of the 16-base fragment, while ddATP had little effect. These results strongly suggest that the 16-base species was indeed the filled-in but unligated intermediate and that fill-in was blocked by wortmannin, presumably as a result of inhibition of the kinase activity of DNA-PK (27), which can regulate accessibility of DNA ends to enzymatic processing (12). In contrast to a resected 3'-PG terminus (10), there was significant removal of PG from the 3'-overhang despite the presence of wortmannin.

In human cell extracts, a slightly different pattern of end joining was seen (Fig. 5). The 3'-overhang substrate yielded primarily the accurate 43-base end joining product, along with variable amounts of the 35- and 37-base products, but none of the 39–42-base products. The 5'-overhang substrate yielded exclusively the accurately joined 37-base product and none of the 35-base resection-dependent product (not shown). In addition, human extracts yielded much more of the 24-, 18-, and 16-base head-to-head end joining products than did hamster and Xenopus extracts, suggesting that there was little or no preference for recircularization over intermolecular end-to-end dimerization. Indeed, analysis on agarose gels indicated that, as reported previously (21), the human cell extracts yielded exclusively intermolecular products, with no detectable recircularization (Fig. 5C). As in the Xenopus extracts, formation of all end joining products was blocked by wortmannin, while substitution of ddTTP for dTTP prevented formation of the accurate 43- and 24-base end joining products and trapped the filled-in but unligated intermediate (16-base fragment in Fig. 5B). Although end joining in human cell extracts was significantly inhibited (about 2-fold) by Me2SO, the inhibition by wortmannin was much greater, ~20-fold.

**DISCUSSION**

The capacity of mammalian cells to join mismatched DNA ends based on short partial complementarities in single strand overhangs was first noticed in the repair joints formed when linearized plasmids with mismatched restriction ends were recircularized upon transfection into monkey CV-1 cells (35). In some of these repair joints, partial complementarities in 3'-overhangs had apparently been used for alignment, despite the
The same requirement would apply to repair of gap-filled staggered 3' overhangs (40). Thus, the use of partial complementarities for alignment of the overhangs during gap filling and ligation would be essential for accurate restoration of the original sequence (Fig. 2). The same requirement would apply to repair of breaks induced by enediyne antibiotics, which involve similar sugar fragmentation on a defined 2- or 3-base 3' stagger (41, 42).

The possibility that Ku might be the long sought “alignment factor” was suggested by its capacity to promote DNA end-to-end association (5–7) and to enhance the joining of either blunt or cohesive DSBs by mammalian DNA ligases (29, 30). The strongest evidence, however, comes from recent analysis of repair joints formed in CHO-K1 and xrs6 extracts during the recircularization of substrates with mismatched restriction ends (8). These data imply a critical role for Ku in preserving 3' overhangs during end joining. Repair joints requiring alignment-based fill-in (see Fig. 2) prior to ligation, in particular, were only formed in extracts from cells expressing functional Ku.

The present results imply a role for Ku in the accurate end joining of staggered free radical-mediated DSBs. For a break formed on a 3-base 3' stagger (giving a 2-base complementarity and a 1-base gap in each strand), a majority, perhaps nearly all, of the end joining events in CHO-K1 extracts reflected complete restoration of the original sequence, whereas in Ku-deficient xrs6 extracts, no accurate joins were detected. Accurate end joining could be restored by the addition of highly purified (25) human Ku to the xrs6 extracts, clearly implicating Ku (rather than, for example, other proteins that might have become either destabilized or overexpressed in Ku-deficient cells) as the critical factor in enforcing the fidelity of end joining. Analogous experiments implicate Ku in preserving the accuracy of joining of a substrate with cohesive 5' overhangs as well, although in this case the requirement for Ku was not absolute. Intriguingly, however, whereas for the 5'-overhang substrate both the accuracy and the efficiency of joining in the Ku-supplemented extract was comparable with that seen in CHO-K1 extract, for the 3'-overhang substrate the efficiency was reduced by more than 30-fold. These results suggest qualitative differences in the precise function of Ku in the joining of the two types of breaks, such that one process is more sensitive to as yet undefined differences between the endogenous hamster and exogenous human Ku.

The internal labeling of the 3'-PG substrates allows intermediate processing of the DNA ends to be analyzed at single-nucleotide resolution, directly demonstrating that, as inferred from analysis of repair joints (8), 3'-end processing and resection are more extensive in Ku-deficient extracts. Nevertheless, the amount of free 3'-hydroxyl-terminated -ACG overhangs available for fill-in and ligation, particularly at short incubation times (data not shown), was comparable in xrs6 and CHO-K1 extracts, yet formation of accurately joined 43-base products in the xrs6 extracts was detectable, at least 50-fold lower than in CHO-K1 (Fig. 3A). This result implies a specific requirement for Ku in alignment during gap filling and/or ligation, in addition to its apparent role in protecting ends from 3' resection. The enzyme that initiates processing by removing the 3'-PG is not known, but it is probably not the apurinic/apyrimidinic endonuclease Apel/Hap1; although Apel1 is the only mammalian enzyme thus far identified that is capable of removing 3'-PGs, it has no detectable activity toward PGs on 3'-overhang substrates (41, 42). Other activities for removing blocking groups from 3' termini have been detected in cell extracts but have not been identified or cloned (45, 46).

Our data, like those derived from substrates with mismatched restriction ends (8), do not support models in which Ku or DNA-PK catalyzes melting of DNA ends, resulting in exposure and annealing of microhomologies within double strand regions (1, 9). On the contrary, end joining events consistent with such annealing were much more frequent in Ku-deficient xrs6 extracts and were completely suppressed by the addition of purified Ku (Fig. 3A). Moreover, in the Xenopus

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**FIG. 5.** End joining in human cell extracts. A, the various substrates were incubated at 37 °C for 6 h, in the presence of 10 μM wortmannin and/or 2.5% MeSO (DMSO) and then cut with XhoI and BstXI. B, same as A, except that certain dNTPs were replaced with ddNTPs as indicated. C, same as A except that substrates and products were analyzed on an agarose gel without restriction cleavage; for clarity, the top portion of the marker lane is shown at 5X reduced contrast.
system, it has been shown that such joining is efficiently catalyzed by extract fractions devoid of Ku and DNA-PKcs (47). Recent in vitro studies (48) suggest that the Mre11 complex is a much more likely candidate for catalyzing end joining based on microhomologies within duplex regions near DNA ends.

DNA-PKcs-deficient XR-C1 extracts, unlike Ku-deficient xrs6 extracts, yielded substantial quantities of accurate 43-base repair products. Moreover, the DNA-PK inhibitor wortmannin had little effect on end joining in CHO-K1 extracts. These results suggest that only Ku and not DNA-PKcs is essential for accurate end joining in vitro. However, in human and Xenopus extracts, end joining of the same substrates is qualitatively similar but is wortmannin-sensitive. Although the wortmannin data are to some extent complicated by the effects of Me2SO alone (a finding that emphasizes the need for concurrent solvent controls whenever this inhibitor is used), we consistently find nearly complete suppression of end joining by wortmannin and other DNA-PK inhibitors in these extracts. While the disparity in wortmannin sensitivity could be due to technical factors such as the differences in preparation of the various extracts, it could reflect the levels of DNA-PKcs.

DNA-PK activity is typically 50–100-fold lower in hamster and other rodent cell extracts than in human cell or Xenopus egg extracts (49–52), and electrophoresis mobility shift assays as well as Western blot assays are consistent with this difference being due to a much lower level of DNA-PKcs protein in rodent cells (53), including CHO cells. Thus, a hypothesis consistent with all of the available data is that although DNA-PKcs is not required for accurate end joining in vitro, sufficient quantities of it produce an inhibitory effect that can be alleviated by activation of its kinase activity.

Recent chemical cross-linking studies (54), as well as physical-chemical data on the binding and activation of Ku and DNA-PKcs (55, 56), suggest that when Ku recruits DNA-PKcs (55, 56), suggest that when Ku recruits DNA-PKcs and Ku, or some other protein in the complex) might then promote either DNA-PKcs dissociation or sliding of the whole complex along the aligned DNA ends, thus allowing Ku to move to its proper position bridging the ends. Phosphorylation of both Ku70 and Ku80 during end joining has been detected in Xenopus egg extracts (52), although in similar experiments we have detected only Ku80 phosphorylation. Thus, in this model, the role of DNA-PKcs is primarily regulatory, initially displacing Ku and thus controlling the timing of end processing through specific phosphorylations, the details of which remain to be defined.

The apparent dispensability of DNA-PKcs in accurate end joining in CHO-K1 extracts does not necessarily imply that it plays no important role in end joining in intact rodent cells. Indeed, at least some rodent cells lacking DNA-PKcs exhibit the same radiosensitivity and DSB repair deficiency as those lacking Ku (4). One possible explanation for this disparity is that DNA-PKcs may play a role in bringing broken DNA ends together, a task that might be much more difficult and complex in intact cells than in in vitro assays, where free ends are relatively abundant. Consistent with this proposal, DNA-PKcs has been reported to promote intermolecular ligation by ligase IV plus XRCC4, while inhibiting intramolecular ligation (58). However, the fact that intramolecular recircularization dominates end joining in Xenopus extracts but does not occur at all in human extracts, despite an apparent abundance of DNA-PKcs in both (49–52), remains to be explained.

Alternatively, recent in situ fluorescence labeling studies suggest that DNA-PK may be largely or at least partly responsible for the phosphorylation of histone H2AX, an event that occurs within minutes of irradiation (59). Localization of phosphorylated H2AX at putative DSB repair foci precedes the colocalization of Bren1, hRad51, and/or the hMre11-hRad50-NBS complex at the same foci, suggesting that H2AX may recruit these DSB repair factors to sites of multiple or difficult-to-repair breaks. Thus, DNA-PK helix enzyme may play a critical role in initiating a complex choreography that serves to optimize repair by assigning broken ends to specific repair pathways (60–62), some of which probably do not utilize Ku for end alignment. Indeed, a defect in DNA-PK activation rather than in end alignment could be the primary reason for the radiosensitivity of Ku-defective cells. The availability of a stringent reconstitution assay (Fig. 3A) may facilitate the identification of Ku mutants differentially defective in each of these two functions and thus allow assessment of their relative importance in DSB repair efficiency, repair fidelity, and radiosensitivity. The assay might also be useful in determining what domains and biochemical properties of Ku70 and Ku80 are required for particular steps in the alignment-based end joining pathway.

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Fidelity of DNA End Joining

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