Nonhomologous end-joining (NHEJ) repairs DNA double-strand breaks created by ionizing radiation and V(D)J recombination. To repair the broken ends, NHEJ processes noncompatible ends into a ligatable form, but suppresses processing of compatible ends. It is not known how NHEJ controls polymerase and nuclease activities to act exclusively on noncompatible ends. Here, we analyzed processing independently of ligation by using a two-stage assay with extracts that recapitulated the properties of NHEJ in vivo. Processing of noncompatible ends required wortmannin-sensitive kinase activity. Since DNA-PKcs brings the ends together before undergoing activation of its kinase, this suggests that processing occurred after synapsis of the ends. Surprisingly, all polymerase and most nuclease activity required XRCC4/Ligase IV. This suggests a mechanism for how NHEJ suppresses processing to optimize the preservation of DNA sequence.

Nonhomologous end-joining (NHEJ) preserves chromosomal integrity by repairing DNA double-strand breaks. Processing of DNA ends is a key step in NHEJ, since double-strand breaks often create ends that are not directly ligatable. For example, ionizing radiation generates nucelotides with aberrant structures such as 3’ phosphate or 3’ phosphoglycolate groups, which must be removed by nuclease activity before ligation can occur.

Processing of DNA ends in vivo has been studied in the context of V(D)J recombination. The RAG1/RAG2 dimer cleaves two sites in the immunoglobulin locus to create two hairpin coding ends and two blunt signal ends (1). Nuclease activity opens the hairpin ends to leave 3’ overhangs, which are filled in to generate P-nucleotide addition (2). To fill in 3’ overhangs, a polymerase must synthesize DNA from a primer on the opposing end. Nucleotide deletion also occurs, but nuclease activity is limited to less than 20 nucleotides, often back to regions of microhomology (3,4). Strikingly, the blunt signal ends are joined without processing. Thus, the NHEJ reaction is strongly biased towards the preservation of DNA sequence, suppressing processing if it is not needed, and limiting the extent of processing when it is required.

The NHEJ reaction requires core proteins and processing enzymes (5). The core proteins include Ku, DNA-PKcs, XRCC4/Ligase IV (XL), and Cernunnos (also named XRCC4-like factor, XLF) (6,7). Proposed processing enzymes include the nuclease Artemis (8), and DNA polymerases mu and lambda (9-12). The biochemical properties of these proteins suggest that NHEJ repairs double-strand breaks in an ordered series of steps. Ku binds to DNA ends and translocates inward, recruiting DNA-PKcs (13,14). DNA-PKcs brings the ends together, activating its kinase activity (15). DNA-PKcs then phosphorylates itself and its target proteins (16). The ends are processed if necessary, and XL catalyzes the final ligation step (17).

Important questions remain unanswered. NHEJ requires DNA-PKcs kinase activity (18), but it is not known whether the kinase activity affects processing or ligation. XL interacts with pol mu, pol lambda, and Cernunnos/XLF (6,7), but it is not known whether XL has a function other than ligation. Furthermore, it is not known how NHEJ suppresses processing of compatible ends.

The biochemistry of processing can be studied in cell free systems that permit manipulation of the component proteins. Progress has been made by partially reconstituting the joining reaction with purified proteins. Nick
McElhinny et al. studied polymerase activity with polymerases mu and lambda acting in conjunction with Ku and XL (19), but their reconstituted system did not include nuclease activity. Ma et al. studied processing by Artemis, polymerases mu and lambda, DNA-PKcs, Ku, and XL (20), but their system lacked Cernunnos/XLF. Furthermore, they observed frequent non-templated nucleotide addition, which does not occur in vivo unless TdT is present (3,4).

We previously reproduced NHEJ in crude cell extracts (21). The joining reaction in extracts had many properties of NHEJ in vivo, including requirements for Ku, DNA-PKcs, and XL. As observed for V(D)J recombination in vivo, polymerase activity was capable of filling in both 5’ and 3’ overhangs; nuclease activity was limited to less than 20 nucleotides and extended back to regions of microhomology. Finally, processing was highly efficient for noncompatible ends, but strongly suppressed for compatible ends.

Here, we used cell-free extracts in a two-stage assay to determine if DNA-PKcs kinase activity or XL is involved in processing or ligation. The assay demonstrated that kinase activation is required for both processing and ligation of DNA ends. In addition, we discovered that XL is required for all of the polymerase activity and much of the nuclease activity in the NHEJ reaction. These results suggest a model for how processing is controlled in vivo.

EXPERIMENTAL PROCEDURES

Extract preparation

We prepared whole cell extracts as previously described (21-23) from pellets of 10 L suspension cultures of HeLa S3 cells grown at the National Cell Culture Center (Minneapolis, MN), and lymphoblastoid cells (GM00558C, Coriell Cell Repositories) grown in medium consisting of RPMI 1640, 15% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 1% penicillin/streptomycin (Gibco).

To immunodeplete XRCC4/Ligase IV, we pre-incubated extract at 4°C for 90 min with XRCC4 antibody (Serotec) at a ratio of 1 µL antibody to 15 µL extract, incubated the mixture with washed protein A-Sepharose beads (Santa Cruz Biotech) at 4°C for 90 min with rotation, and centrifuged the mixture at 4000 g for 5 min, leaving the supernatant for the NHEJ reaction. To inhibit the kinase activity of DNA-PKcs, we pre-incubated extract with 10 µM wortmannin for 30 min on ice.

DNA substrates

We used PCR to amplify DNA fragments DNA1 (869 bp) and DNA2 (819 bp) with primers containing restriction enzyme sites for EcoRI, BamHI, KpnI, EcoRV, Sacl, PvuI, or ClaI as previously described (21). Direct digestion of the PCR products could produce contamination from residual uncut DNA. Therefore, we subcloned the PCR products into the pCR-BluntII-TOPO vector (Invitrogen), released the DNA fragment by cleavage with the appropriate restriction enzyme, and purified DNA1 and DNA2 by agarose gel electrophoresis.

Two-stage assay for processing of DNA ends

In Stage 1, we incubated 4 µL extract at 37°C for 90 minutes with DNA fragments, DNA1 and DNA2, each at a concentration of 18.4 fmol, in a 20 µL volume containing 50 mM Tris-Cl pH 8.0, 60 mM KOAc, 0.5 mM MgCl2, 1 mM ATP, 1 mM DTT, 0.08 mg/ml BSA, as previously described (21). Some reactions contained 25 µM dNTPs, as indicated. We started reactions by adding extract to the DNA and buffer mixture, and stopped reactions by adding 2 µL of 0.5 M EDTA, pH 8.

In Stage 2, we purified the DNA by loading the reaction mixture from Stage 1 onto a QiaQuick column (Qiagen PCR purification kit), and eluting the DNA products in 100 µL buffer containing 10 mM Tris-Cl pH 8.5. We incubated half of the DNA overnight at room temperature with 1 µL T4 DNA Ligase (1 Weiss Unit/µL; USB Corporation) in the manufacturer’s buffer, and then purified the DNA on a QiaQuick column. As a control, we incubated half of the DNA in manufacturer’s buffer only. We chose T4 Ligase from USB Corporation because it contained lower levels of contaminating nuclease activity than several other commercial preparations.

We performed each joining reaction in triplicate. To avoid jackpot effects, we harvested DNA products from at least two independent joining reactions, amplified the DNA by PCR, and
sequenced five DNA junctions as previously described (21). The primer termini were located 182 and 583 bases from the ends of DNA1 and DNA2, respectively, to permit detection of large deletions. Joining efficiency was measured by quantitative PCR as previously described (21). “Percentage of DNA ends joined” was based on a reference sample for 100% joining, which consisted of 18.4 fmol of preformed junction DNA. Quantitative PCR detected only the junctions specified by the PCR primers. The 3’ ends of the quantitative PCR primers were 109 and 132 bases from the ends of DNA1 and DNA2, respectively, and the 3’ end of the TaqMan probe was 104 bases from the end of DNA2.

**Purification of recombinant XRCC4/Ligase IV**

Baculoviruses expressing recombinant XRCC4 and Ligase IV (a gift from Dr. William Dynan) were used to co-infect 1 L of Sf9 insect cells at a multiplicity of infection of 2. About 50 h post-infection, cells were harvested by centrifugation, flash-frozen, and stored at -80°C. Cells were thawed, resuspended in 35 ml of lysis buffer (20 mM NaH2PO4 pH 7.4, 0.5 M NaCl, 10 mM imidazole, 1% IGEPAL-CA630, 5 mM β-ME, 1 mM phenylmethanesulfonyl fluoride, 2.7 mM benzamidine, EDTA-free protease inhibitor cocktail tablet [Roche]), lysed by sonication, and cleared by centrifugation at 100,000g for 1 h.

To purify recombinant XL, we incubated the supernatant with 0.5 ml Ni2+NTA-agarose beads (Qiagen) in the presence of 20 mM imidazole for 1 h at 4°C, packed the beads into a column, washed the column several times with wash buffer (20 mM NaH2PO4, 0.5 M NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol, 10% glycerol) at alternating pHs of 7.4 and 6.0, and eluted proteins with wash buffer at pH 7.4 containing 300 mM imidazole. We analyzed fractions for the presence of rXL by SDS-PAGE and immunoblot, purified the rXL fractions on a second column consisting of Superdex 200 (26/60) (Amersham Pharmacia Biotech) pre-equilibrated with S buffer (50 mM Tris pH 7.4, 0.5 M NaCl, 1 mM EDTA, 10% glycerol, 10 mM β-mercaptoethanol), dialyzed peak fractions against S buffer containing 20 mM NaCl, and purified XL further on a third column consisting of Mono Q (5/50 GL) (Amersham Pharmacia Biotech) pre-equilibrated with S buffer containing 20 mM NaCl, eluting rXL with a linear gradient of NaCl (0.02 M to 0.6 M) in S buffer. Fractions containing rXL were flash-frozen and stored at -80°C in aliquots at a final concentration of 2 µM.

For use in the NHEJ reaction, we diluted the purified rXL 20-fold with extract buffer (20 mM Tris-Cl pH 8.0, 100 mM KOAc, 0.5 mM EDTA, 1 mM DTT, 20% glycerol), and added 1 µL of diluted rXL to the 20 µL end-joining reaction, so that the final concentration of rXL was 5 nM. Comparative Western blots showed that the XL concentration in extracts was 1-2 nM (data not shown).

**RESULTS**

A two-stage assay for productively processed ends

Cell extracts can process and ligate DNA ends in a manner that recapitulates NHEJ in vivo (21). Joining requires Ku, XL, and DNA-PKcs. However, it is not known if XL or DNA-PKcs participates in the processing step or the ligation step of NHEJ.

We used a two-stage assay to separate the processing and ligation steps (Fig. 1A). For Stage 1, we prepared linear DNA substrates, DNA1 and DNA2 by cleaving the ends on each substrate with one of several restriction enzymes: EcoRI, KpnI, SacI, or EcoRV (21). We then incubated the DNA substrates with one of four extract preparations: (1) untreated extract; (2) extract treated with wortmannin to inhibit the kinase activity of DNA-PKcs; (3) extract immunodepleted with anti-XRCC4 antibodies to remove XL; and (4) XL-depleted extract supplemented with purified recombinant XL (rXL).

For Stage 2, we purified DNA from the joining reaction and identified productively processed ends that remained unligated by exploiting the ability of T4 Ligase to join compatible ends (24). Thus, we treated half of the purified DNA with T4 DNA Ligase, and left the other half of the DNA untreated (Fig. 1A). We then measured joining efficiency by quantitative PCR, and examined the processed ends by PCR amplification and sequencing of the junctions.

*T4 Ligase can be used to detect ligatable ends generated during the two-stage assay*
We wanted to ensure that T4 Ligase could detect a mixture of processed ends, since extracts often process a pair of noncompatible DNA ends in several different ways (21). To mimic heterogeneous processing by extracts, we created substrates by cleavage with a different restriction enzyme at each end (Fig. 1B).

We first tested T4 Ligase on one pair of compatible ends created from BamHI, EcoRI, KpnI, or SacI. With these substrates, T4 Ligase joined compatible ends with efficiencies of 12% to 15% (Fig. 1B). By contrast, the maximum joining efficiency was 33%, since T4 Ligase potentially joined the new substrates in 6 orientations, and the PCR primers detected only 2 of the orientations (Supplemental Fig. 1). Thus, T4 Ligase joined a large fraction of the compatible ends in a homogeneous DNA population of DNA1 and DNA2 molecules.

We then tested T4 Ligase on mixtures of compatible ends. Joining efficiency decreased less than 2-fold when the mixture contained 2 or even 4 different substrates (Fig. 1B). Thus, the two-stage assay with T4 Ligase was capable of detecting a large fraction of productively processed ends, even in a heterogeneous mixture.

To confirm that T4 Ligase could detect productively processed ends with high specificity, we compared ligation of compatible ends to ligation of noncompatible ends. T4 Ligase joined compatible ends 1000-fold more efficiently than noncompatible ends (Supplemental Fig. 2). The low levels of joining for noncompatible ends were due to contamination of the commercial T4 Ligase preparations with nuclease activity, which converted the overhanging ends into blunt ends (data not shown). Thus, T4 Ligase showed high specificity for the detection of compatible ends.

**HeLa and lymphoblastoid extracts recapitulate NHEJ in vivo**

We performed the two-stage assay on extracts from HeLa and lymphoblastoid cells to ensure that our conclusions would not be specific for a single cell type. Thus, extracts from 2 cell lines were treated in 4 ways (Fig. 1A), with and without added dNTPs. In Stage 1, extract preparations were incubated with one of 3 pairs of ends. In Stage 2, the DNA products were left untreated or treated with T4 Ligase. To facilitate comparisons, experiments included all 96 reactions executed in parallel, using a single pool of reagents (Supplemental Fig. 3). The experiments were performed in triplicate.

For all experiments with extracts, each DNA substrate was created with a single restriction enzyme. Extracts joined these substrates by intermolecular ligation, without detectable intramolecular ligation into circular monomers (21). The maximum joining efficiency for extracts was 12.5%, since extracts potentially joined ends in 16 orientations, and the PCR primers detected only 2 orientations (Supplemental Fig. 1).

Extracts from HeLa and lymphoblastoid cells processed noncompatible ends using polymerase and nuclease activities (Fig. 2 and Supplemental Fig. 4). Polymerase activity filled in both 5' and 3' overhangs, as observed in vivo, and was greatest in extracts with efficient joining. For example, HeLa extract with added dNTPs joined EcoRI-KpnI ends with a high efficiency of 6.38%, and 10 of 20 strands were processed by nucleotide addition (Fig. 2). When dNTPs were omitted, joining efficiencies in HeLa and lymphoblastoid extracts decreased 30 and 56-fold for 5'-3' noncompatible ends (EcoRI-KpnI), and 8 and 6-fold for blunt-3' noncompatible ends (EcoRI-Sacl), respectively (Fig. 3). Thus, most joining required polymerase activity.

Nuclease activity in extracts resembled the nuclease activity observed in vivo. Deletions were limited to less than 20 nucleotides, often extending back to regions of microhomology. Nuclease activity was partially obscured by the polymerase activity in HeLa extracts, but was more readily observed in lymphoblastoid extracts, particularly without added dNTPs. For example, when dNTPs were omitted from lymphoblastoid extracts, 13 of 20 strands from EcoRI-KpnI ends were processed by nucleotide deletion (Fig. 2).

NHEJ in vivo requires DNA-PKcs kinase activity and XL. Our NHEJ reaction in vitro had the same requirements. Joining was blocked by wortmannin (Fig. 3, compare “Ext, untreated” to “Ext + wort”, T4 Ligase omitted in Stage 2). Joining was also blocked by immunodepletion with anti-XRCC4 antibodies (Fig. 3, compare “Ext, untreated” to “Ext – XL”, T4 Ligase omitted in Stage 2). In summary, extracts had properties of NHEJ in vivo, including appropriate polymerase and nuclease activities as well as requirements for XL and DNA-PKcs kinase activity.
Extracts suppress processing of compatible ends

HeLa and lymphoblastoid extracts joined compatible EcoRI-EcoRI ends with efficiencies of 3.2% to 8.9% (Fig. 3, upper panels, “Ext, untreated”). These efficiencies are quite high when compared to the maximum of 12.5%. Extracts joined the compatible ends precisely, with virtually no evidence of processing. For this report, we sequenced a total of 80 junctions generated from compatible ends (EcoRI-EcoRI) (Supplemental Fig. 3I). In a previous report, we sequenced 52 junctions generated from compatible ends (KpnI-KpnI and BamHI-BamHI) (21). Processing affected only one of these 132 junctions. Thus, extracts suppressed virtually all processing when the ends could be ligated directly.

Extracts process and join noncompatible ends with high efficiency

HeLa extracts with dNTPs joined noncompatible ends as efficiently as compatible ends (Fig. 3A, middle and lower panels, “Ext, untreated”), even though joining noncompatible ends was significantly more complex, requiring the recruitment of polymerase and nuclease activities. To match the efficiency for compatible ends, the NHEJ reaction must have processed most of the noncompatible ends into a ligatable form.

Lymphoblastoid extracts joined noncompatible ends with 50-fold lower efficiency than HeLa extracts, and processed the ends with less polymerase activity (Fig. 3B, middle and lower panels, “Ext, untreated”). Thus, a DNA polymerase, or a factor required for DNA polymerase activity, appeared to be deficient in the lymphoblastoid extracts. When dNTPs were omitted, joining efficiencies decreased, albeit more markedly in lymphoblastoid than in HeLa extracts (Fig. 3C and 3D, middle and lower panels, “Ext, untreated”). Thus, joining efficiencies for noncompatible ends were consistently lower in lymphoblastoid extracts than in HeLa extracts.

To determine whether extracts processed but failed to join some of the ends, we added T4 Ligase in Stage 2. However, T4 Ligase did not affect joining efficiency for noncompatible ends (Fig. 3, middle and lower panels, “Ext, untreated”). Thus, when extracts processed DNA ends into a compatible form, they almost always succeeded in joining the ends. This result raised the possibility that processing might be coupled to ligation.

Kinase activity is required for processing and ligation of DNA ends

To examine the role of kinase activity in the processing and ligation steps of NHEJ, we treated extracts with wortmannin to inhibit kinase activity. For both compatible and noncompatible ends, wortmannin decreased joining efficiency at least 100-fold in HeLa and lymphoblastoid extracts (Fig. 3, “Ext + wort”). For noncompatible ends, wortmannin blocked reactions dominated by polymerase activity, such as joining of 5’ overhanging EcoRI to 3’ overhanging KpnI ends in HeLa extracts (Fig. 3A, middle panel). Wortmannin also blocked reactions in lymphoblastoid extracts dominated by nuclease activity, such as joining of 5’ overhanging EcoRI to 3’ overhanging KpnI ends (Fig. 3D, middle panel) or joining of blunt EcoRV to 3’ overhanging SacI ends (Fig. 3D, lower panel). Addition of T4 Ligase in Stage 2 failed to increase the percentage of ends joined (Fig. 3, middle and lower panels, “Ext + wort”). Thus, kinase activity was required for the processing of noncompatible ends.

Compatible ends remained unprocessed when kinase activity was inhibited by wortmannin. Addition of T4 Ligase in Stage 2 restored full joining efficiency (Fig. 3, upper panels, compare “Buffer” and “Ext + wort”), and the junctions were joined precisely. Wortmannin permits DNA-PKcs to bind DNA ends (15,25), but prevents DNA-PKcs from undergoing autophosphorylation and releasing the ends (26-29). These observations suggest that wortmannin-treated DNA-PKcs remained bound to the DNA ends in our extracts, blocking access by the processing enzymes.

XL depletion reduces processing during NHEJ

To investigate the role of XL in the processing and ligation steps of NHEJ, we removed XL from extracts by immunodepletion with anti-XRCC4 antibody. More than 98% of XL was successfully removed, as determined by immunoblotting (data not shown).

For compatible ends, XL depletion decreased joining efficiency at least 1000-fold (Fig. 3, upper panels, “Ext – XL”), consistent with the fact that XL is the only known ligase for...
NHEJ. Upon addition of T4 Ligase in Stage 2, joining efficiency increased up to the level of joining by T4 Ligase alone, and the junctions were joined precisely. Thus, the compatible ends remained unprocessed in the absence of XL. This raised the possibility that the presence of XL might be required for processing to occur.

For noncompatible ends, XL depletion decreased joining efficiency to the background levels for buffer alone (Fig. 3, middle and lower panels, compare “Buffer” to “Ext – XL”). Furthermore, extracts disrupted by XL depletion (or wortmannin inhibition) often processed the noncompatible ends to produce large deletions (Fig. 3 and Supplemental Fig. 3). Most of these deletions were greater than 100 nucleotides and represented an unregulated nuclease activity. On the other hand, intact extracts rarely processed input strands with deletions greater than 20 nucleotides. Thus, junctions with large deletions were products of an inefficient reaction extrinsic to the bona fide NHEJ reaction.

Deletions larger than 109 bases from the end of DNA1 or 104 bases from the end of DNA2 did not contribute to our measurement of joining efficiency because of the location of the quantitative PCR probe and primers (Experimental Procedures). Quantitative PCR did detect some junctions with deletions greater than 20 bases (e.g., 2 junctions in Supplemental Fig. 3A, “Ext – XL”, left column; one junction in Supplemental Fig. 3E, “Ext + wort”, left column). Thus, the actual joining efficiencies were even lower than the measured joining efficiencies when NHEJ was inhibited by wortmannin or XL-depletion.

Addition of T4 Ligase in Stage 2 after XL depletion in Stage 1 consistently increased joining efficiency, albeit to a low level of 0.003% to 0.02% of the input DNA ends. We sequenced a total of 40 junctions recovered by T4 Ligase from XL-depleted extracts. Of the 160 DNA strands from 40 junctions, 87 strands were processed by nuclease activity (77 strands had deletions less than 20 nucleotides), and only 4 strands were processed by polymerase activity (Fig. 3). Therefore, low levels of processing in the absence of XL consisted almost exclusively of nuclease activity.

Addition of rXL to XL-depleted extracts restores NHEJ

To ensure that the effects of immunodepletion were due to loss of XL, we purified recombinant XRCC4/Ligase IV (rXL) from baculovirus-infected insect cells (Supplemental Fig. 2). Purified rXL had no detectable ligase activity for noncompatible ends in our NHEJ buffer conditions.

When we added rXL to XL-depleted extracts, joining was indistinguishable from joining by intact extracts (Fig. 3, middle and lower panels, compare “Ext, untreated” to “Ext – XL + rXL”). Intact extracts and XL-depleted extracts supplemented with XL showed the same polymerase and nuclease activities, including 3’ and 5’ fill-in polymerase activities, and 3’ and 5’ nuclease activities (Fig. 4). When we examined processing in greater detail, there were no statistically significant differences (Supplemental Fig. 5). Therefore, the defects in XL-depleted extracts were due to the loss of XL, and not due to the loss of another factor that was removed during the immunodepletion.

XL is required for all polymerase activity and most nuclease activity

To assess the effect of XL on processing, we analyzed each NHEJ reaction in terms of net polymerase and nuclease activities (Fig. 5). These activities were expressed as the percentage of input strands with additions or deletions. Net polymerase or nuclease activity was computed by multiplying joining efficiency by the fraction of strands processed by additions or deletions, respectively.

As an example, we will show our calculation of net nuclease activity for EcoRI-KpnI ends incubated with XL-depleted lymphoblastoid extract without added dNTPs and with T4 Ligase added in Stage 2. Joining efficiency for the reaction was 0.0027%. Among the 5 sequenced junctions (Supplemental Fig. 3G, right panel, “Ext – XL”), one junction contained large deletions in DNA2 that excluded it from the NHEJ reaction and from the measurement of joining efficiency. Among the 4 remaining junctions, 7 strands were unprocessed, and 9 were processed by limited deletions. Thus, 9 of 16 strands were processed by regulated nuclease activity, and net nuclease activity was \((9/16) \times 0.0027\%\), or 0.0015% of the input strands.

As noted previously, the junctions
recovered from intact extracts and XL-depleted extracts supplemented with rXL were indistinguishable, with or without T4 Ligase in Stage 2. To permit a more accurate estimate of the data, junctions from these four reactions were combined and referred to as junctions produced by “complete extract”. Our conclusions did not change when each reaction condition was examined separately.

We first examined the effect of XL on polymerase activity (Fig. 5, upper panel). When XL was depleted from extracts, the percentage of input strands with additions was less than 0.0002% and did not increase upon treatment with T4 Ligase in Stage 2. Therefore, essentially all polymerase activity required XL.

The effect of XL on nuclease activity was more complex (Fig. 5, lower panel). When XL was depleted from extracts, processing by nucleotide deletion occurred for less than 0.0002% of the input strands. Addition of T4 Ligase in Stage 2 consistently increased the percentage of input strands processed by nuclease activity. In lymphoblastoid extract without dNTPs, the increased levels of nuclease activity approached that of complete extracts (44% to 75% of the activity in complete extracts). However, these extract preparations had very low joining efficiencies and thus very low levels of net nuclease activity.

For other extract preparations, the nuclease activity recovered by addition of T4 Ligase in Stage 2 fell far short of the nuclease activity in complete extracts. In NHEJ reactions with the highest efficiencies, 97% to 99% of the nuclease activity required XL (Fig. 5, HeLa extract + dNTPs). Therefore, in the full NHEJ reaction, most nuclease activity required XL.

**DISCUSSION**

*Role of the DNA-PKcs kinase in processing and ligation of DNA ends*

NHEJ occurs only if the DNA-PKcs kinase domain is intact (18). However, it is not known which steps in the joining reaction require the kinase. To address this, we inhibited the kinase activity of DNA-PKcs by adding wortmannin to extracts.

To distinguish among the wortmannin-sensitive kinases in the PI3-kinase family, we added wortmannin in titrated concentrations. The IC50 of wortmannin was 200 nM for both inhibition of purified DNA-PKcs and inhibition of NHEJ in extracts (data not shown). The IC50 of wortmannin is 5 nM for PI3-kinase (30,31), 100 to 150 nM for ATM (30,32), and 1800 nM for ATR (30). Therefore, the effects of wortmannin on NHEJ in extracts were attributable to inhibition of DNA-PKcs or ATM, but not PI3-kinase or ATR.

ATM plays a role in the resolution of double-strand breaks, since loss of ATM leads to a defect in V(D)J recombination associated with formation of inappropriate hybrid junctions between coding and signal ends (33). ATM appears to stabilize newly created DNA ends and thus prevent illegitimate joining events. This would suppress formation of hybrid junctions during V(D)J recombination and chromosomal translocations from sporadic double-strand breaks. Nevertheless, the hybrid junctions have hallmarks of NHEJ, including template-dependent nucleotide addition and nucleotide deletion back to regions of microhomology. Since appropriate processing and ligation occur during formation of the hybrid junctions, ATM is not involved in the processing and ligation steps of NHEJ. Therefore, the effects of wortmannin on NHEJ in extracts are most likely due to inhibition of DNA-PKcs kinase activity.

The two-stage assay revealed that wortmannin blocked processing by both polymerase and nuclease activities (Fig. 3). Thus, processing required kinase activity. DNA-PKcs has biochemical properties consistent with a role in facilitating the processing of DNA ends. DNA-PKcs autophosphorylation induces a conformational change to release the ends (26-29). This may expose the DNA ends to the processing enzymes. Indeed, DNA-PKcs autophosphorylation facilitates Artemis endonuclease activity (34). DNA-PKcs is also required for polynucleotide kinase to convert ends with 5' OH groups into 5'-phosphates (24). Thus, DNA-PKcs kinase activity is required for processing by Artemis nuclease and polynucleotide kinase.

DNA-PKcs kinase activity appears to be required for ligation as well as processing, since wortmannin inhibited the joining of compatible ends in the NHEJ reaction. Inhibition of ligation was not due to inappropriate processing of the compatible ends, since virtually all of the ends remained viable substrates for T4 Ligase in the
two-stage assay. Inhibition was also not due to a direct effect on XL. In the presence of 5% PEG, purified rXL ligated a modest percentage (0.06%) of compatible ends in the absence of other NHEJ factors, and wortmannin did not affect this ligation activity (data not shown). On the other hand, the effect of wortmannin on ligation activity in extracts was consistent with observations that DNA-PKcs autophosphorylation releases the DNA ends, which would then be available for ligation. Taken together, our experiments suggest that DNA-PKcs kinase activity is required for both processing and ligation in the NHEJ reaction.

**XL ensures that processing of DNA ends occurs only if needed for joining**

We were surprised to discover that removal of XL from extracts abolished all polymerase and most nuclease activity. When recombinant XL was added to the XL-depleted extracts, processing was fully restored. We conclude that XL is required for efficient processing of noncompatible ends.

All processing by polymerase activity required XL. Interestingly, DNA polymerases mu and lambda associate with Ku and XL in vitro (19,20,35). Interaction between XL and DNA polymerase mu facilitates the association of both proteins with DNA (9).

Fill-in of 3’ overhanging ends occurred frequently during the processing of noncompatible ends, and represented all the polymerase activity in the joining of EcoRV-Sacl blunt-3’ ends (Fig. 2 and Supplemental Fig. 4). Indeed, Ku and XL may align the DNA ends so that polymerase mu can fill in 3’ overhangs by priming from the opposing DNA end (19). As expected, omission of dNTPs suppressed most polymerase activity (Fig. 5).

However, significant levels of polymerase activity persisted in the absence of dNTPs. For example, HeLa extracts without dNTPs processed 0.26% of the input ends by nucleotide addition, which was 11% of the polymerase activity with dNTPs added (Fig. 5, top panel). These data might be explained by the presence of residual dNTPs after dialysis, or by production of dNTPs from nuclease activity during the NHEJ reaction. However, the data also raise the possibility that extracts contain a single-strand ligase activity. In extracts without added dNTPs, the single-strand ligase would create a continuous DNA strand.

Subsequent PCR amplification for analysis of the joining reaction would generate apparent fill-in of 3’ overhangs. The putative single-strand ligase activity would require XL, since XL-depletion removed polymerase activity (Fig. 5, top panel). Supporting this possibility, a weak single-strand ligase activity has been reported for XL (20).

Our data suggest a model in which DNA-PKcs recruits XL to the DNA ends prior to the processing reactions. In fact, DNA-PKcs forms a complex with XL at DNA ends and stimulates the ligation activity of XL (36). This explains why compatible ends are almost never processed. XL joins the ends before the processing enzymes have a chance to modify the ends. For noncompatible ends, the presence of XL during the processing steps ensures immediate ligation once the ends become compatible. Indeed, intact extracts joined all productively processed ends, since T4 Ligase failed to detect residual processed ends. Thus, NHEJ appears to couple processing and ligation, which may play a role in limiting the extent of the processing reactions.

**Control of processing by DNA-PKcs and XL**

The NHEJ reaction controls processing to preserve DNA sequence. This occurs during V(D)J recombination in vivo, and occurred during NHEJ in our extracts. Compatible DNA ends were joined without processing, and noncompatible DNA ends were processed in an efficient and regulated manner.

How does NHEJ control processing? The first step of the joining reaction is likely to be the binding of Ku to the DNA ends (Fig. 6). Ku is abundant, has a high affinity for DNA ends, and its binding to DNA ends is readily detected in an electrophoretic mobility shift assay (37). Ku then recruits DNA-PKcs to the ends (13,14). Cells lacking Ku or DNA-PKcs fail to join coding ends during V(D)J recombination, and the residual junctions contain large deletions apparently catalyzed by an unregulated nuclease activity (38). Thus, the binding of Ku and DNA-PKcs appears to physically protect the ends from nucleases.

DNA-PKcs brings the ends together into a synaptic complex, and only then is the kinase activated (15). Prior to synopsis, DNA-PKcs remains bound to the DNA ends, preventing processing and ligation. This gatekeeper function of DNA-PKcs ensures that processing occurs only
after the DNA ends have been juxtaposed to each other. Activation of the DNA-PKcs kinase leads to autophosphorylation and a conformational change that makes the ends accessible to XL and the processing enzymes (26-29).

We propose a model for how NHEJ controls the processing of DNA ends after DNA-PKcs autophosphorylation (Fig. 6). In a minor pathway, nuclease binds and processes the ends into a compatible form independently of XL. Recruitment of nuclease for this minor pathway may be mediated by DNA-PKcs, which interacts with Artemis (8). The endonuclease activity of Artemis is consistent with much of the processing that acts on overhanging ends in XL-depleted extracts (Supplemental Fig. 3, “Ext – XL”, with T4 Ligase in Stage 2).

In the major pathway of the model, DNA-PKcs recruits XL to the ends (36). DNA-PKcs undergoes autophosphorylation, and joining occurs immediately if the ends are compatible (Fig. 6). If the ends are not compatible, our data demonstrate that XL must be present for processing to occur. We hypothesize that XL is an integral component of the synaptic complex containing the DNA ends and processing proteins. As a result, ligation would occur as soon as the ends are properly processed.

Processing appears to occur within a synaptic complex containing the DNA ends. For example, nuclease activity removed nucleotides from both ends back to regions of microhomology shared by the two ends. Polymerase and nuclease activities often acted on opposing ends to make them compatible for ligation (Fig. 2 and Supplemental Fig. 3). Similar processing events also occur during V(D)J recombination in vivo. The presence of XL at the DNA ends would provide a mechanism for minimizing such processing and preserving the integrity of the genome.

REFERENCES

1. van Gent, D., Ramsden, D., and Gellert, M. (1996) Cell 85, 107-113
2. Schlissel, M. (1998) Mol Cell Biol 18, 2029-2037
3. Gilfillan, S., Dierich, A., Lemuer, M., Benoist, C., and Mathis, D. (1993) Science 261, 1175-1178
4. Komori, T., Okada, A., Stewart, V., and Alt, F. (1993) Science 261, 1171-1175
5. Weterings, E., and van Gent, D. C. (2006) Cell 124(2), 301-313
6. Ahnesorg, P., Smith, P., and Jackson, S. P. (2006) Cell 124(2), 301-313
7. Buck, D., Malivert, L., de Chasseval, R., Barraud, A., Fondaneche, M. C., Sanal, O., Plebani, A., Stephan, J. L., Huflagel, M., le Deist, F., Fischer, A., Durandy, A., de Villartay, J. P., and Revy, P. (2006) Cell 124(2), 287-299
8. Ma, Y., Pannicke, U., Schwarz, K., and Lieber, M. R. (2002) Cell 108, 7995-8006
9. Bebenek, K., Garcia-Diaz, M., Blanco, L., and Kunkel, T. A. (2003) J Biol Chem 278(36), 34685-34690
10. Capp, J. P., Boudsocq, F., Bertrand, P., Laroche-Clary, A., Pourquier, P., Lopez, B. S., Cazaux, C., Hoffmann, J. S., and Canitrot, Y. (2006) Nucleic Acids Res 34(10), 2998-3007
11. Hammarsten, O., and Chu, G. (1998) Proc Natl Acad Sci USA 95, 525-530
12. Yoo, S., and Dynan, W. S. (1999) Nucleic Acids Res 27(24), 4679-4686.
13. DeFazio, L., Stansel, R., Griffith, J., and Chu, G. (2002) EMBO J. 21, 3192-3200
14. Meek, K., Gupta, S., Ramsden, D. A., and Lees-Miller, S. P. (2004) Immunol Rev 200, 132-141
15. Grawunder, U., Wilm, M., Xiantuo, W., Kulezla, P., Wilson, T. E., Mann, M., and Lieber, M. R. (1997) Nature 388, 492-494
16. Kurimasa, A., Kumano, S., Boubnov, N., Story, M., Tung, C. S., Peterson, S., and Chen, D. (1999) Mol. Cell. Biol. 19, 3877-3884
17. Nick McElhinny, S. A., Havener, J. M., Garcia-Diaz, M., Juarez, R., Bebenek, K., Kee, B. L., Blanco, L., Kunkel, T. A., and Ramsden, D. A. (2005) Mol Cell 19(3), 357-366
20. Ma, Y., Lu, H., Tippin, B., Goodman, M. F., Shimazaki, N., Koiwai, O., Hsieh, C. L., Schwarz, K., and Lieber, M. R. (2004) Mol Cell 16(5), 701-713
21. Budman, J., and Chu, G. (2005) EMBO J. 24(4), 849-860
22. Baumann, P., and West, S. C. (1998) Proc Natl Acad Sci USA 95, 14066-14070
23. Budman, J., and Chu, G. (2006) Methods Enzymol 408, 430-444
24. Chappell, C., Hanakahi, L. A., Karimi-Busheri, F., Weinfeld, M., and West, S. C. (2002) EMBO J 21(11), 2827-2832
25. Calsou, P., Frit, P., Humbert, O., Muller, C., Chen, D. J., and Salles, B. (1999) J. Biol. Chem. 274(12), 7848-7856
26. Ding, Q., Reddy, Y. V., Wang, W., Woods, T., Douglas, P., Ramsden, D. A., Lees-Miller, S. P., and Meek, K. (2003) Mol Cell Biol 23(16), 5836-5848
27. Reddy, Y. V., Ding, Q., Lees-Miller, S. P., Meek, K., and Ramsden, D. A. (2004) J Biol Chem 279(38), 39408-39413
28. Weterings, E., Verkaik, N. S., Bruggenwirth, H. T., Hoeijmakers, J. H., and van Gent, D. C. (2003) Nucleic Acids Res 31(24), 7238-7246
29. Block, W. D., Yu, Y., Merkle, D., Gifford, J. L., Ding, Q., Meek, K., and Lees-Miller, S. P. (2004) Nucleic Acids Res 32(14), 4351-4357
30. Sarkaria, J. N., Tibbetts, R. S., Busby, E. C., Kennedy, A. P., Hill, D. E., and Abraham, R. T. (1998) Cancer Res 58(19), 4375-4382.
31. Okada, T., Sakuma, L., Fukui, Y., Hazeki, O., and Ui, M. (1994) J Biol Chem 269(5), 3563-3567
32. Chan, D. W., Son, S. C., Block, W., Ye, R., Khanna, K. K., Wold, M. S., Douglas, P., Goodarzi, A. A., Pelley, J., Taya, Y., Lavin, M. F., and Lees-Miller, S. P. (2000) J Biol Chem 275(11), 7803-7810
33. Bredemeyer, A. L., Sharma, G. G., Huang, C. Y., Helmink, B. A., Walker, L. M., Khor, K. C., Nuskey, B., Sullivan, K. E., Pandita, T. K., Bassing, C. H., and Sleckman, B. P. (2006) Nature 442(7101), 466-470
34. Goodarzi, A. A., Yu, Y., Riballo, E., Douglas, P., Walker, S. A., Ye, R., Harer, C., Marchetti, C., Morrice, N., Jeggo, P. A., and Lees-Miller, S. P. (2006) EMBO J. 25(16), 3880-3889
35. Fan, W., and Wu, X. (2004) Biochem Biophys Res Commun 323(4), 1328-1333
36. Chen, L., Trujillo, K., Sung, P., and Tomkinson, A. E. (2000) J Biol Chem 275(34), 26196-26205.
37. Rathmell, W. K., and Chu, G. (1994) Proc. Natl. Acad. Sci. USA 91, 7623-7627
38. Taccioli, G., Rathbun, G., Oltz, E., Stamato, T., Jeggo, P., and Alt, F. (1993) Science 260, 207-210

FIGURE LEGENDS

Fig. 1. Assay for roles of kinase activity and XRCC4/Ligase IV in processing DNA ends. (A) Two-stage assay for processing by extracts. DNA molecules with different internal sequences (DNA1 and DNA2) were created by cleavage with various restriction enzymes. The figure shows DNA1 and DNA2 with noncompatible ends consisting of blunt EcoRV and 3’ overhanging SacI ends, respectively. In Stage 1, the DNA substrates with or without added dNTPs were incubated with one of four extract preparations: untreated; treated with wortmannin (Ext + wort); immunodepleted with anti-XRCC4 antibodies (Ext – XL); or immunodepleted and supplemented with recombinant XL (Ext – XL + rXL). In Stage 2, the DNA was purified and analyzed for the presence of compatible ends by incubation with or without T4 Ligase. (B) T4 Ligase detects a heterogeneous mixture of processed ends. DNA substrates were prepared with ends created by restriction enzymes with 5’ overhanging (BamHI or EcoRI) or 3’ overhanging (KpnI or SacI). The other end of each substrate was created with a different restriction enzyme (PvuI for DNA1 and Clal for DNA2) to prevent intramolecular ligation into a circular monomer.

Fig. 2. HeLa and lymphoblastoid extracts show variation in NHEJ. DNA substrates with noncompatible EcoRI-KpnI ends were incubated with HeLa or lymphoblastoid extract with or without added dNTPs. The legend indicates joining efficiency to the right of the arrow. The number of times each junction was recovered is shown in the column labeled “No.” Processing was catalyzed by nuclease activity (open-
mouthed icon and white background) and polymerase activity (arrow and black background). The right column indicates the number of DNA strands processed by nucleotide addition, deletion, or deletion greater than 20 bases. The numbers of strands appear in boxes shaded according to the key above the right column. Supplemental Fig. 4 shows data for joining of noncompatible EcoRV-Sacl ends. Polymerase activity dominated processing by HeLa extract, while nuclease activity was more prominent in processing by lymphoblastoid extract.

**Fig. 3.** Wortmannin treatment and XL depletion inhibit processing of noncompatible DNA ends. In Stage 1, DNA substrates with the indicated ends were incubated with (A) HeLa extract with added dNTPs; (B) HeLa extract without added dNTPs; (C) lymphoblastoid extract with added dNTPs; or (D) lymphoblastoid extract without added dNTPs. When indicated, the extract was treated with wortmannin (Ext + wort) or immunodepleted with anti-XRCC4 antibody (Ext – XL). To confirm the specificity of the immunodepletion, recombinant XRCC4/Ligase IV (rXL) was added to the reactions (Ext – XL + rXL). In Stage 2, the DNA from Stage 1 was incubated with T4 DNA ligase or a buffer control. Joining of EcoRI-EcoRI compatible ends was less efficient for buffer + T4 Ligase than for extract ± T4 Ligase, because ligation into circular monomers occurred for T4 Ligase, but not extract (20). The data supporting this figure appear in Supplemental Fig. 3.

**Fig. 4.** XL-depleted extracts rescued with recombinant XL exhibit normal processing of DNA ends. Noncompatible DNA ends were incubated with HeLa or lymphoblastoid extract with or without added dNTPs. Since T4 Ligase had no significant effect on processing or joining in these reactions, data were combined from experiments in which T4 Ligase was absent or present in the Stage 2 reaction. The graph shows the number of DNA strands processed by polymerase and nuclease activities on the 3’ and 5’ terminal strand. There were no significant differences between untreated extract (Ext) and XL-depleted extract supplemented with rXL (Ext – XL + rXL), since even the largest differences had p-values (p = .36 and p = .57) that failed to reach statistical significance. These data are analyzed in more detail in Supplemental Fig. 5.

**Fig. 5.** XL is required for all of the polymerase activity and much of the nuclease activity at noncompatible DNA ends. Noncompatible DNA ends were incubated with HeLa or lymphoblastoid extract with or without added dNTPs. The graph shows polymerase and nuclease activities at the indicated DNA ends after incubation with XL-depleted extract, XL-depleted extract followed by T4 Ligase, or “complete extract”. The data for complete extract were calculated from an average of the data for extract (Ext) and XL-depleted extract supplemented with rXL (Ext – XL + rXL) with and without T4 Ligase in Stage 2. To calculate the percentage of input strands with additions or deletions, the joining efficiency was multiplied by the fraction of DNA strands showing additions or deletions. The small number of DNA strands with deletions greater than 20 bases were attributed to a background unregulated nuclease activity and omitted from this analysis.

**Fig. 6.** Mechanism for the control of processing during NHEJ. Ku binds to DNA ends and recruits DNA-PKcs, which mediates synopsis of the ends. Synapsis activates DNA-PKcs kinase activity. DNA-PKcs recruits XRCC4/Ligase IV to the DNA ends, undergoes autophosphorylation, and releases the DNA ends (large arrow). XL joins the ends if they are compatible. If the ends are not compatible, XL remains in the synaptic complex to facilitate processing of the DNA ends. Cernunnos/XLF (C/X) plays a role in NHEJ that may be mediated by its interaction with XL. As soon as the ends are processed into a compatible substrate, XL completes the joining reaction. In a minor pathway (small arrow), nuclease processes the DNA ends independently of XL.
Figure 2

| EcoRI-KpnI (5'-3' noncompatible ends) |
|--------------------------------------|
| EcoRI                                 |
| TGCCAAAG | AATT |
| ACGGTTCTTAA |  |
| KpnI                                 |
| 5' CGAGAT |
| 3' CATGGCTCATA |
| DNA junctions                          |
| Input DNA ends                        |
| % ends joined                         |

| HeLa ext | + dNTPs | (6.38%) |
|----------|---------|---------|
| TGCCAAAG | AATT | +4 | 4 |
| ACGGTTCTTAA | 0 | 0 | 0 |
| +4 | 1 |

| HeLa ext | - dNTPs | (0.21%) |
|----------|---------|---------|
| TGCCAAAG | AATT | +4 | 4 |
| ACGGTTCTTAA | 0 | 0 | 0 |
| -2 | 1 |

| Lymphoblastoid ext | + dNTPs | (0.14%) |
|--------------------|---------|---------|
| TGCCAAAG | 0 | +3 | 5 |
| ACGGTT | -5 | 0 | 0 |

| Lymphoblastoid ext | - dNTPs | (0.0025%) |
|--------------------|---------|---------|
| TGCCAAAG | 0 | +3 | 3 |
| ACGGTT | -5 | 0 | 0 |
| TGCC | -3 | 1 |
| AC | -9 | 1 |
| TGCA | -2 | 1 |

| # of strands | Addition | Deletion | Del 20 |
|--------------|----------|----------|--------|
|              | 10       | 1        | 0      |
|              | 10       | 1        | 0      |
|              | 5        | 5        | 0      |
|              | 3        | 13       | 0      |
### Figure 3

#### A  HeLa extract + dNTPs

| Stage 1 | Stage 2 (T4 Lig) | Percentage of DNA ends joined | # of strands |
|---------|------------------|-------------------------------|--------------|
|         |                  | 10^-3 | 10^-2 | 10^-1 | 1 | 10 | 100 | 1-3 | 7-9 | 10-20 |
| Buffer  | -                | 1.4   |       |       |   |    |     |     |     |       |
|         | +                | 6.8   |       |       |   |    |     |     |     |       |
| Ext, untreated | -      | 5.3   |       |       |   |    |     |     |     |       |
|         | +                | 6.8   |       |       |   |    |     |     |     |       |
| Ext + wort | -      | 0.0022 |       |       |   |    |     |     |     |       |
|         | +                | 1.3   |       |       |   |    |     |     |     |       |
| Ext - XL | -                | 0.0047 |       |       |   |    |     |     |     |       |
|         | +                | 1.8   |       |       |   |    |     |     |     |       |
| Ext - XL + rXL | -      | 7.1   |       |       |   |    |     |     |     |       |
|         | +                | 5.9   |       |       |   |    |     |     |     |       |

#### B  Lymphoblastoid extract + dNTPs

| Stage 1 | Stage 2 (T4 Lig) | Percentage of DNA ends joined | # of strands |
|---------|------------------|-------------------------------|--------------|
|         |                  | 10^-3 | 10^-2 | 10^-1 | 1 | 10 | 100 | 1-3 | 7-9 | 10-20 |
| Buffer  | -                |       |       |       |   |    |     |     |     |       |
|         | +                |       |       |       |   |    |     |     |     |       |
| Ext, untreated | -      |       |       |       |   |    |     |     |     |       |
|         | +                |       |       |       |   |    |     |     |     |       |
| Ext + wort | -      |       |       |       |   |    |     |     |     |       |
|         | +                |       |       |       |   |    |     |     |     |       |
| Ext - XL | -                |       |       |       |   |    |     |     |     |       |
|         | +                |       |       |       |   |    |     |     |     |       |
| Ext - XL + rXL | -      |       |       |       |   |    |     |     |     |       |
|         | +                |       |       |       |   |    |     |     |     |       |

#### Additional Information

- **EcoRI-EcoRI (5'-5' comp)**
- **EcoRI-KpnI (5'-3' noncomp)**
- **EcoRV-SacI (3'-3' noncomp)**
Figure 3

C HeLa extract – dNTPs

| Stage 1 | Stage 2 (T4 Lig) | Percentage of DNA ends joined | # of strands |
|---------|------------------|-------------------------------|--------------|
|         |                  | 10^{-3} | 10^{-2} | 10^{-1} | 1 | 10 | 100 |
| Buffer  | -                | 0.14    | 3.2     | 1.4    | 0.001 | 0.029 | 3.0 |
| Ext, untreated | -         | 0.21    | 3.5     | 3.5    | 0.001 | 0.029 | 3.0 |
| Ext + wort | -           | 0.18    | 3.2     | 1.4    | 0.001 | 0.029 | 4.1 |
| Ext - XL | -                | 0.14    | 1.2     | 1.2    | 0.001 | 0.029 | 4.1 |
| Ext - XL + rXL | -        | 0.18    | 1.2     | 1.2    | 0.001 | 0.029 | 4.1 |

D Lymphoblastoid extract – dNTPs

| Stage 1 | Stage 2 (T4 Lig) | Percentage of DNA ends joined | # of strands |
|---------|------------------|-------------------------------|--------------|
|         |                  | 10^{-3} | 10^{-2} | 10^{-1} | 1 | 10 | 100 |
| Buffer  | -                | 0.14    | 3.2     | 1.4    | 0.001 | 0.029 | 3.0 |
| Ext, untreated | -         | 0.21    | 3.5     | 3.5    | 0.001 | 0.029 | 3.0 |
| Ext + wort | -           | 0.18    | 3.2     | 1.4    | 0.001 | 0.029 | 4.1 |
| Ext - XL | -                | 0.14    | 1.2     | 1.2    | 0.001 | 0.029 | 4.1 |
| Ext - XL + rXL | -        | 0.18    | 1.2     | 1.2    | 0.001 | 0.029 | 4.1 |
Figure 4

|            | EcoRV-Sacl (Bl-3') | EcoRI-KpnI (5'-3') |
|------------|--------------------|--------------------|
| **HeLa + dNTPs** | ![Graph](image) | ![Graph](image) |
| **HeLa - dNTPs** | ![Graph](image) | ![Graph](image) |
| **Lymphoblastoid + dNTPs** | ![Graph](image) | ![Graph](image) |
| **Lymphoblastoid - dNTPs** | ![Graph](image) | ![Graph](image) |

Number of DNA strands with indicated enzymatic activity.

Polymersase | Nuclease | Polymersase | Nuclease
Figure 5

Polymerase activity

- Ext - XL
- Ext - XL (+T4)
- Complete Ext

Nuclease activity

- EcoRI-KpnI
- EcoRV-Sacl

Lymphoblastoid

HeLa
Figure 6

Ku and DNA-PKcs bind the ends

synapsis of the ends activates the kinase

DNA-PKcs undergoes autophosphorylation, releasing the ends

XL binds the ends

nuclease binds the ends

XL joins the ends if compatible, OR...

XL facilitates processing

polymerase

nuclease

XL binds the ends

XL joins the ends
Processing of DNA for nonhomologous end-joining is controlled by kinase activity and XRCC4/LigaseIV
Joe Budman, Sunny A. Kim and Gilbert Chu

*J. Biol. Chem.* published online January 31, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M610058200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2007/03/09/M610058200.DC1