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Ku Stimulation of DNA Ligase IV-dependent Ligation Requires Inward Movement along the DNA Molecule*

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The DNA ligase IV·XRCC4 complex (LX) functions in DNA non-homologous-end joining, the main pathway for double-strand break repair in mammalian cells. We show that, in contrast to ligation by T4 ligase, the efficiency of LX ligation of double-stranded (ds) ends is critically dependent upon the length of the DNA substrate. The effect is specific for ds ligation, and LX/DNA binding is not influenced by the substrate length. Ku stimulates LX ligation at concentrations resulting in 1–2 Ku molecules bound per substrate, whereas multiply Ku-bound DNA molecules inhibit ds ligation. The combined footprint of DNA with Ku and LX bound is the sum of each individual footprint suggesting that the two complexes are located in tandem at the DNA end. Inhibition of Ku translocation by the presence of *cis*-platinum adducts on the DNA substrate severely inhibits ligation by LX. Fluorescence resonance energy transfer analysis using fluorophore-labeled Ku and DNA molecules showed that, as expected, Ku makes close contact with the DNA end and that addition of LX can disrupt this close contact. Finally, we show that recruitment of LX by Ku is impaired in an adenylation-defective mutant providing further evidence that LX interacts directly with the DNA end, possibly via the 5′-phosphate as shown for prokaryotic ligases. Taken together, our results suggest that, when LX binds to a Ku-bound DNA molecule, it causes inward translocation of Ku and that freedom to move inward on the DNA is essential to Ku stimulation of LX activity.

DNA non-homologous end-joining (NHEJ)[1] is the major mechanism for the repair of DNA double-strand breaks in mammalian cells and rejoins breaks induced during V(D)J recombination. Five proteins function directly in the end-joining process in mammalian cells (1, 2), namely DNA ligase IV and XRCC4, and the DNA-dependent protein kinase complex (DNA-PK), which comprises two subunits of the Ku heterodimer and a large catalytic subunit, DNA-PKcs. Recently, Artemis has also been shown to play some role in the DNA NHEJ process (3, 4).

DNA ligase IV has a conserved ligase domain at its N terminus and two BRCT domains at its C terminus. DNA ligase IV forms a tight complex with XRCC4 (LX) via an interaction that requires the region between the two BRCT domains (5). Recently, mutations in DNA ligase IV were found in patients displaying radiosensitivity, immunodeficiency, and developmental delay (6). Although LX plays a unique role in NHEJ, it has been reported to be an inefficient double-stranded (ds) ligase and specifically is less effective than either of the two more abundant mammalian ligases, DNA ligases I and II (7–10). However, using similar molar ratios of enzyme and substrate to those used in studies reporting inefficient ds ligation by LX, we found that baculovirus-expressed LX is a highly efficient ds ligase (7–11). Here, we examine the basis underlying these contradictory findings and show that LX has specific substrate length requirements. Using longer substrates than those used previously, we show that the LX complex is a highly efficient ds ligase. These differences are not mediated by either the ability of LX to interact with substrates of different length nor with its ability to form a DNA·adenylate complex.

Ku has been shown to nonspecifically stimulate ds ligation, a feature attributable in part to its ability to juxtapose two DNA ends (8, 12, 13). Additionally, Ku has been reported to help recruit LX to DNA ends (14). Conversely, Ku has also been reported to inhibit LX activity (15). The recently solved crystal structure of a Ku·DNA complex shows that Ku forms an open ring-like shape with the DNA threaded through the aperture (16). This structure allows Ku to interact with two turns of the DNA, but access to the DNA ends remains allowing additional protein-DNA interactions. Ku also has the ability to translocate along the DNA molecule (Refs. 17 and 18; reviewed in Ref. 19).

Having established the parameters for efficient ds ligation by LX, we examined the impact of Ku on ligation. We show that, although Ku stimulates ligation when only 1 or 2 Ku heterodimers are bound to the substrate, multiply bound Ku molecules inhibit ligation. Using a range of biochemical and bio-

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[1] The abbreviations used are: NHEJ, non-homologous end-joining; DNA-PK, DNA-dependent protein kinase complex; ds, double strand; LX, DNA ligase IV·XRCC4 complex; FRET, fluorescence resonance energy transfer; EMSA, electrophoretic mobility shift assay; cisplatin, *cis*-platinum; TCSPC, time-correlated single photon counting; PEG, polyethylene glycol; AF488, Alexa Fluor 488; AF564, Alexa Fluor 564.

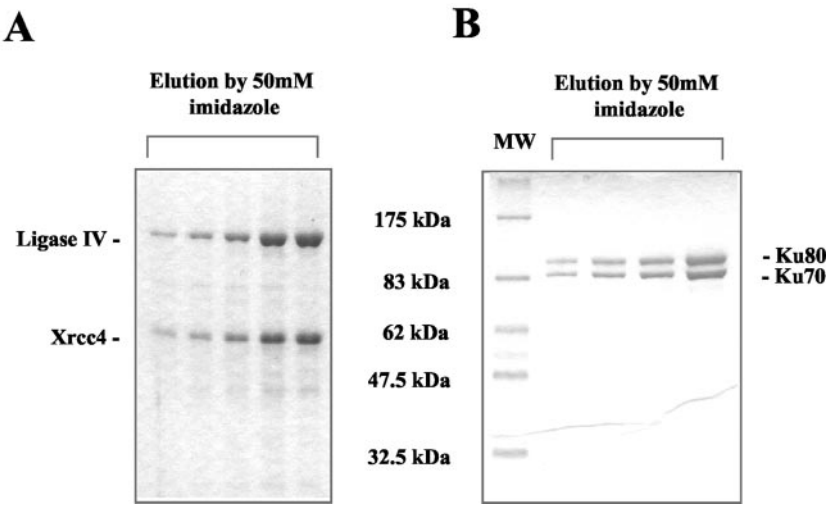

FIG. 1. **Purification of Ku70/80 and LX complexes.** *A*, purification of the LX complex; *B*, purification of the Ku70/80 heterodimer on Talon affinity resin to near homogeneity.

physical techniques, including footprinting analysis and fluorescence resonance energy transfer (FRET), we show that the recruitment of LX by Ku requires movement of proteins along the DNA molecule, disruption of the tight binding of Ku to the DNA ends, and an adenylated LX complex. Taken together, our results suggest a model in which Ku binds to the DNA end, recruits LX, and translocates inwardly on the DNA molecule to allow direct interaction of LX with the DNA end and subsequent ligation.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant LX and Ku Complexes—Both protein complexes were overexpressed in the Bac-To-Bac™ baculovirus expression system (Invitrogen) and purified to near homogeneity on metal-chelate Talon affinity resin and Mono Q ion-exchanger as described previously (11, 20).

Nick Ligation Assay—Nick ligation was performed as described previously (21). Briefly, indicated amounts of LX or T4 ligase were incubated with 70 fmol of nicked substrate in 20 μl of reaction mixture for 1 h. In all the analyses, dried gels were analyzed and quantified using a Storm PhosphorImager (Amersham Biosciences).

ds Ligation Assay—3 pmol of LX and T4 ligase was incubated for 2 h in a 30-μl reaction mixture with 70 fmol of DNA as described previously (11). dsDNA fragments were produced from the Bluescript plasmid (Stratagene, La Jolla, CA) to give substrates of 53 and 445 bp with 4-bp overhangs at each end, and a 157-bp substrate with 4- and 2-bp overhangs, using *Pst*I, *Afl*II, *Kpn*I, and *Pvu*II restriction enzymes, respectively. Reactions with Ku were preincubated for 15 min on ice with the indicated amounts of Ku, and ligation was initiated by addition of the enzyme and transfer to 37 °C.

EMSA—For EMSA studies, LX was incubated with the DNA in 10 mM triethanolamine (pH 7.5), 0.5 mM dithiothreitol, 0.1 mg/ml bovine serum albumin at 4 °C for 1 h. Loading buffer was added (20% Ficoll, 0.4% xylene cyanol), and the samples were subjected to 8% PAGE in 0.5× TAE buffer (20 mM Tris acetate, 0.5 mM EDTA), pH 8.1, with constant cooling and buffer recircularization. For Ku EMSA, Ku was incubated with the DNA in 50 mM triethanolamine, pH 7.5, 2 mM $Mg(OAc)_2$, 2 mM dithiothreitol, 0.1 mg/ml bovine serum albumin as described above. Following addition of loading buffer, the samples were electrophoresed on a 0.8% agarose gel in 0.5× TBE buffer.

DNase I Footprinting—A 174-bp DNA fragment, an *Eco*RI-*Pvu*II double digest of the plasmid pKS, was labeled at the 3′-end using [γ-^{32}P]dATP and AMV reverse transcriptase. Samples (3 μl) of the labeled DNA fragments (175 fmol) were incubated with 5 μl of the buffered solution containing the relevant protein (0.5 and 1.0 pmol of Ku, 5 and 10 pmol of LX). After 30-min incubation at 37 °C, 2 μl of DNase I (0.01 unit/ml^{-1}) was added. After 1 min, the reaction was stopped by boiling. Samples were processed as standard sequencing reactions.

DNA Platinization—Platinization of DNA was performed as described previously (22). Briefly, 200 ng of a [γ-^{32}P]ATP-labeled, 445-bp *Afl*III-*Pst*I Bluescript fragment was incubated with 0.2 nM cisplatin (Cisplatyl, Bellon) in buffer containing 10 mM $NaHPO_4$ (pH 7.5) and 3 mM NaCl for 16 h at 37 °C in the dark. The optimum cisplatin concentration was determined empirically from preliminary experiments. The DNA was purified from unreacted *cis*-Pt using Qiagen reaction clean-up columns. The radioactivity recovered was determined by liquid scintillation counting of an aliquot of the eluate, and the amount of DNA recovered was calculated on the basis of the specific activity obtained in the original labeling reaction.

Fluorescence Resonance Energy Transfer Analysis—Alexa Fluor 488 dUTP (Molecular Probes, Inc., Eugene, OR) was incorporated into the DNA substrate using a standard terminal transferase labeling protocol, and the gel was purified to remove unincorporated nucleotides from the reaction mixture. 100 μg of the recombinant Ku heterodimer was labeled with an Alexa Fluor 568 monoclonal antibody labeling kit (Molecular Probes, Inc.) and purified following the manufacturer's instructions. The labeled conjugates were stored at 4 °C and protected from light. Control experiments showed that labeling with 2–4 mol of Alexa Fluor 568 dye per mole of Ku heterodimer, which was used in the subsequent experiments, did not alter the DNA binding affinity of Ku (data not shown). The 100-μl reactions were carried out on ice: 5 pmol of Ku was preincubated with 1.75 pmol of DNA for 30 min, followed (where indicated) by the addition of 10 pmol of LX (molar DNA:protein: protein ratios were identical to those used in the footprinting experiments). Time-correlated single photon counting (TCSPC) was performed using a modified multiphoton microscope system (Bio-Rad MRC 1024MP) with the scanning function disabled. TCSPC imaging capability was carried out similarly to that described previously (23), by addition of photomultiplier (Hamamatsu R7401-P) with fast single-photon response in the re-projected stationary plane of the objective and time-correlated single photon counting electronics (Becker & Hickl, SPC 700). Buffer solutions were analyzed on microscope coverslips, imaged with a 40 × 1.3 numerical aperture (Nikon CFi Fluor) oil immersion lens. Fluorescence was excited at 890 nm, and emission was collected at 500 ± 20 nm (Coherent Inc., 35–5040). Laser power was adjusted to give average photon counting rates of the order 10^4 to 10^5 photons s^{-1} (0.0001–0.001 photons per excitation event) and with peak rates approaching 10^6 photons s^{-1}, below the maximum counting rate afforded by the TCSPC card to avoid pulse pile-up. Analysis of the fluorescence transients was performed primarily with the SPCimage software package (Becker & Hickl Gmbh, Berlin) with additional graphical representation and analysis using in-house developed software tools.

Generation of the K273G DNA Ligase IV·XRCC4 Complex—To produce ligase IV protein bearing a single lysine to glycine amino acid substitution at the position 273, the DNA ligase IV gene was amplified from a wild type in pFastBacHTc with two pairs of oligonucleotides as follows (mutated nucleotides are in boldface): in the first amplification, the sense primer 5′-TATTCCGGATTATTCATACCGTCC-3′ and antisense 5′-TTCACCATCACC**GGT**TTCTATGTAGAAACTCTGATG-3′ were used to produce the first fragment (~0.85 kb). In the second amplification, the sense primer 5′-TGTGGTATGGCTGATTATGATCC-C-3′ and antisense primer 5′-TACATAGAAACC**GGT**CTAGATGGTGA-ACGTATGCAA-3′ were used to produce a second fragment (~2.0 kb). The mutations generate a unique *Age*I restriction site, which was used in subsequent cloning as follows: after purification, the first PCR product was digested with *Xho*I/*Age*I and the second with *Bam*HI/*Age*I. The resulting restriction fragments were gel-purified and ligated into pFastBacHTc vector (Invitrogen) linearized with *Xho*I/*Bam*HI. Recom-

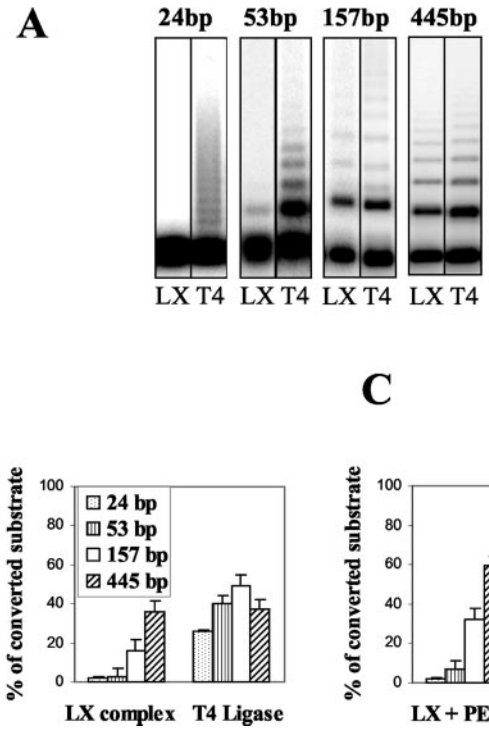

FIG. 2. **Double-stranded ligation efficiencies of LX and T4 DNA ligase using substrates of different lengths.** *A*, ds ligation reaction at equimolar concentrations of the enzymes with substrates of different lengths in the absence of PEG. *B* and *C*, quantitative analysis of these reactions in the absence and presence of 12% PEG for LX and T4 DNA ligase. The amount of enzymes corresponded to mid-point of the linear range of the reactions. For all results, quantitative analysis was carried out using a Storm PhosphorImager and ImageQuaNT software and represents the results of at least three independent experiments.

binant Ligase IV K273G·XRCC4 complex was produced using the baculovirus expression complex as described for the wild type complex.

RESULTS

Overexpression and Purification of LX and Ku70/80 Complexes—Genes for each subunit of both complexes were separately cloned into the Bac-To-Bac™ baculovirus his-tag expression system (Invitrogen) and co-transfected into Sf9 insect cells. Fig. 1 shows typical elution profiles for the Talon affinity-purified Ku and LX complexes.

LX ds Ligation, but Not nick Ligation, Shows Substrate Length Specificity—To examine the impact of substrate length on ligation, we examined the ds ligation activity of LX and T4 ligase using substrates of differing lengths ranging from 24 to 445 bp using equimolar DNA concentrations. LX ligation efficiency ranged from 3 to 40% depending on substrate length (Fig. 2, *A* and *B*). The efficiency was strikingly lower for the short DNA fragments (24 and 53 bp), but good ligation was observed with the 445-bp fragment. In contrast, although T4 ligase showed lower efficiency of ligation with a 24-bp fragment, it did not show any preference for substrate length for fragments above 53 bp demonstrating that the findings are LX-specific and not attributable to ds ligation *per se*. The crowding agent, PEG (12%), stimulated both LX and T4 ligation efficiency (1.5- to 2-fold), but the impact of substrate length remained unchanged (Fig. 2*C*). In the control experiments, no significant difference in the ligation efficiency was observed between the 157-bp fragment of pBluescript plasmid generated by *Kpn*I and *Pvu*II restriction enzymes (2- and 4-bp overhangs) and the 144-bp fragment of the pFastBac1 plasmid generated by *Kpn*III and *Bam*HI restriction enzymes (both 4-bp overhangs; data not shown).

Next, we used an electrophoresis mobility shift assay (EMSA)

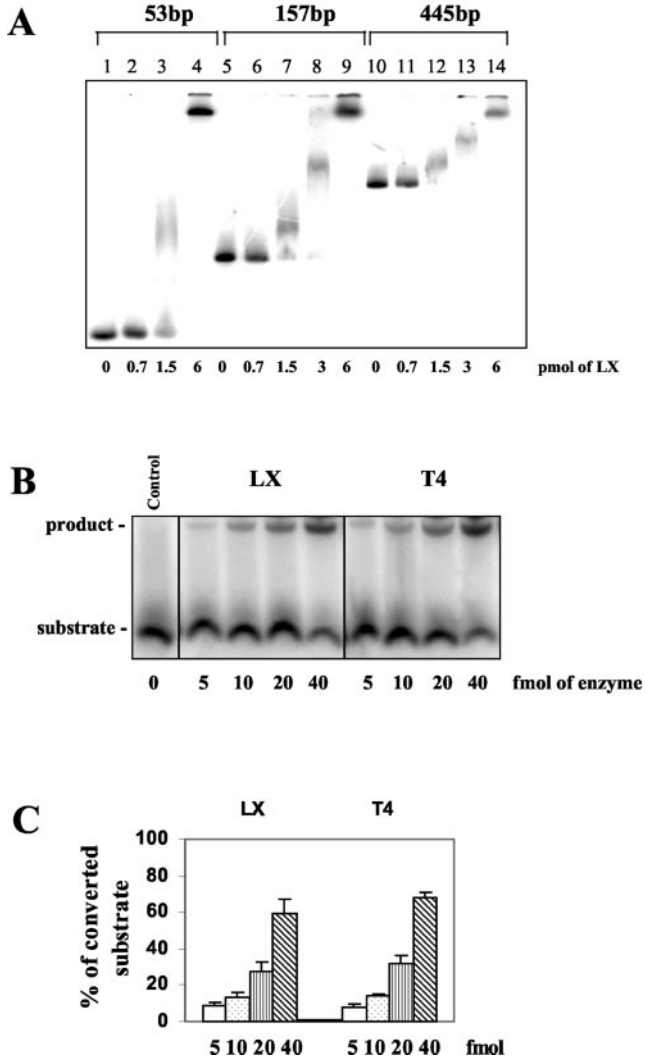

FIG. 3. **DNA binding of the LX complex to the substrates of different lengths and the comparison of nick ligation activities of LX complex and T4 DNA ligase on a synthetic 51-bp-long substrate.** *A*, binding of the LX complex to DNA at increasing concentrations of the complex. Equimolar concentrations of the DNA substrates were used in all reactions. *B* and *C*, capacity of the LX complex to ligate a single-strand-nicked DNA substrate of 51 bp compared with T4 DNA ligase.

to monitor the binding of the LX complex to the three longer DNA substrates. With all three substrates, no binding of the LX complex was observed at low concentrations (0.7 pmol), weak binding indicated by a smeared band shift was observed at intermediate concentrations (1.5–3 pmol), and strong binding occurred at higher LX concentrations (6 pmol) (Fig. 3*A*). The sharp increase in band shift product and the progressive mobility decrease were indicative of a co-operative mode of DNA binding. We also compared the nick ligation activity of the LX complex relative to the activity of T4 ligase using a short (51 bp) substrate. Equimolar concentrations of LX and T4 Ligase showed almost identical nick ligation efficiencies (Fig. 3, *B* and *C*) in marked contrast to the differing ds ligation efficiency on a 53-bp substrate (Fig. 2, *A* and *B*). Taken together, these findings support the conclusion that, although LX can bind efficiently to a short DNA substrate, can form a DNA adenylate complex and seal nicks, it exhibits a strong preference for substrates of 157 and 445 bp for ds ligation, a feature not observed with T4 ligase.

Impact of Ku on LX Ligation Using Different Length Substrates—Previous studies have demonstrated that the Ku70/80

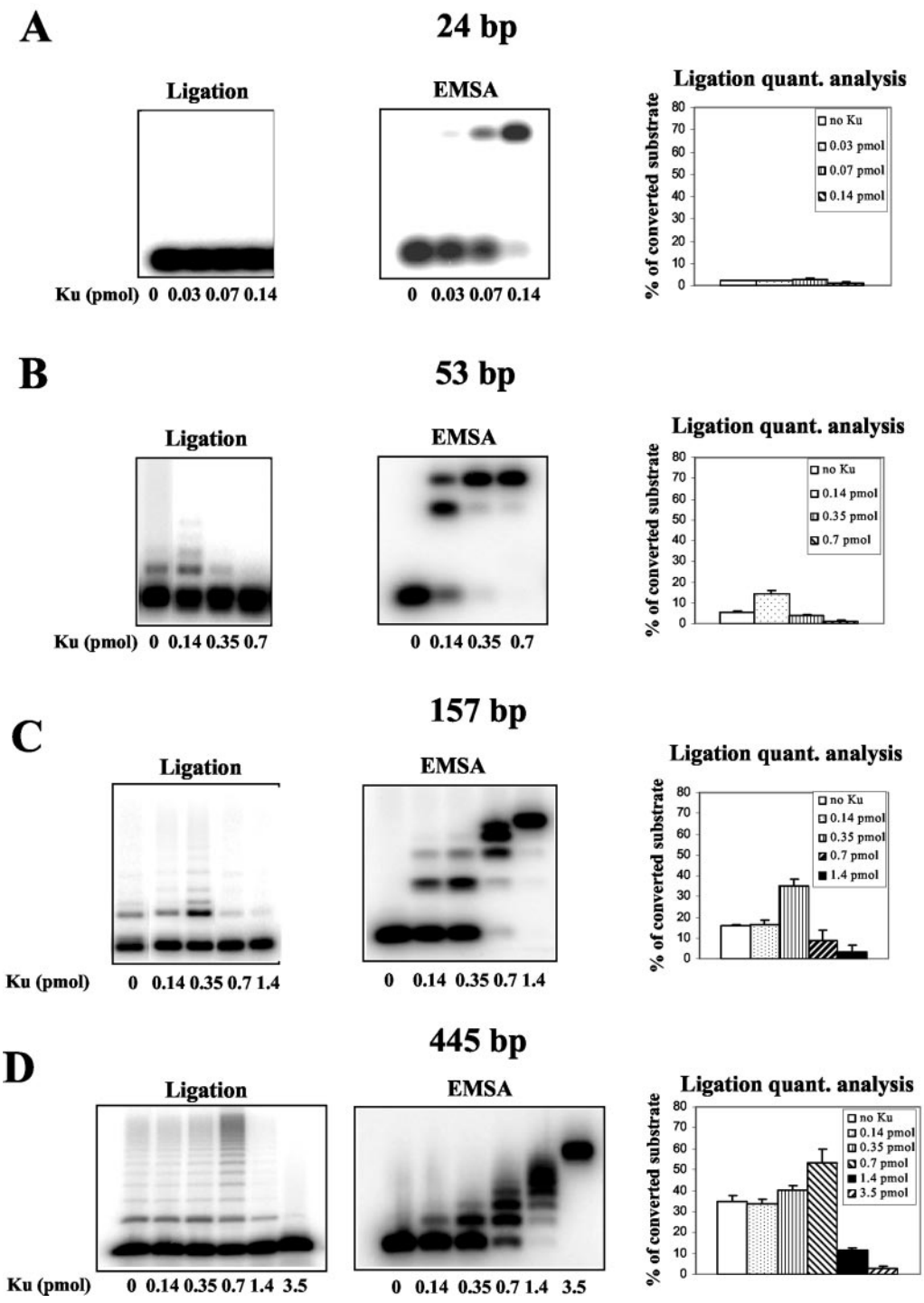

FIG. 4. **Ku70/80 can stimulate as well as inhibit double-stranded ligation.** The impact of increasing molar ratios of Ku70/80 to DNA on ds ligation was assessed on all four DNA substrates using 3 pmol of the LX complex (*A*, 53 bp; *B*, 157 bp; *C*, 445 bp). Stimulation of ligation was only observed at a Ku concentration corresponding to that at which the majority of the substrate molecules were bound by one or two Ku heterodimers (compare corresponding lanes in *Ligation* and *EMSA panels*). Higher concentrations of Ku resulted in inhibition of ligation.

heterodimer is able to stimulate LX ligation activity, although distinct but convincing studies have, in contrast, demonstrated an inhibition of LX ligation by Ku (8, 15). We, therefore, examined the impact of the Ku heterodimer on LX ligation using substrates of different lengths. Because the impact of Ku may depend upon substrate length, we carried out the reactions at differing concentrations of Ku. For the 24-bp substrate no ligation activity was observable even following the addition of Ku (Fig. 4*A*). For the other substrates, stimulation of ligation by Ku was observed over a narrow range of Ku to DNA molar ratios with inhibition being observed at higher concentrations of Ku (Fig. 4, *left* and *right panels*). We next used EMSA to establish the number of Ku molecules bound to each substrate at the concentrations used for ligation. A distinct band shift pattern was obtained with each substrate with the number of Ku molecules bound increasing proportionally to the length of DNA substrate (Fig. 4, *middle panels*). Comparison of ligation and EMSA shows that stimulation of ligation occurs only at Ku concentrations corresponding to 1–2 Ku heterodimers bound to the majority of substrate molecules. A relatively modest increase in Ku concentration, resulting in more DNA substrate being bound by multiple Ku molecules, significantly inhibited

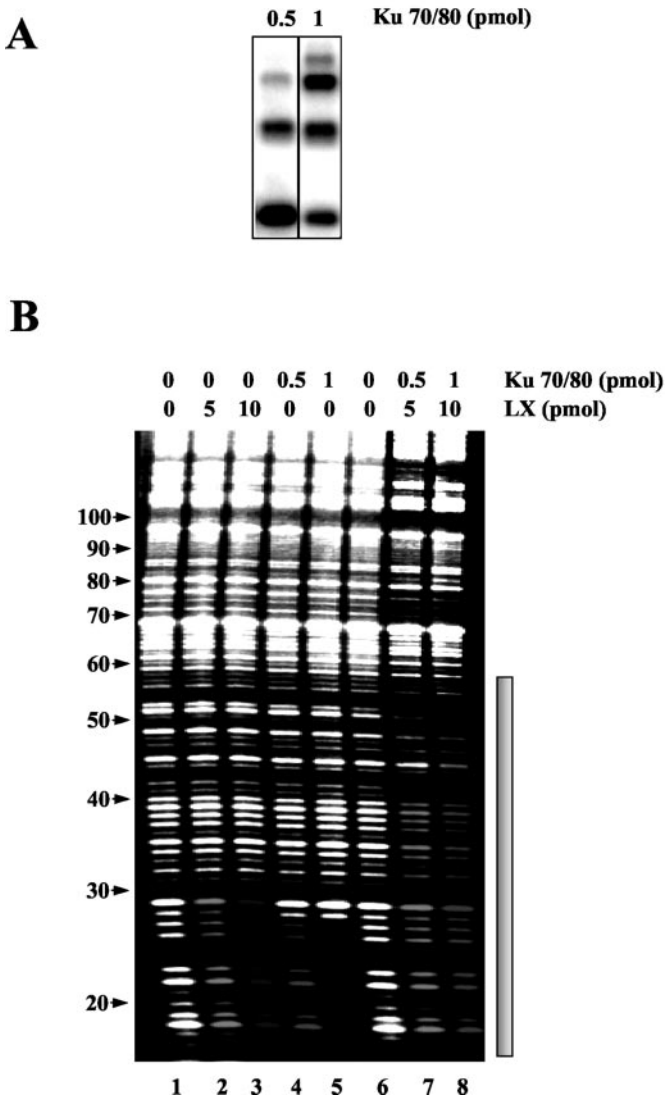

FIG. 5. **Footprinting analysis of DNA-bound Ku and LX complexes.** DNA footprinting analysis was used to assess the mode of binding of Ku70/80 and LX individually and in combination. *A*, a control band-shift experiment. In *B*: *lane 1*, control lane with no added proteins; *lanes 2* and *3*, LX individual footprints with 5 and 10 pmol of LX, respectively; *lanes 4* and *5*, Ku individual footprints with 0.5 and 1 pmol of Ku, respectively; *lane 6*, control lane with no added proteins; *lanes 7* and *8*, the combined footprint of LX and Ku (10 pmol of LX and 1 pmol of Ku).

ligation even at concentrations of Ku non-saturating for binding. Nearly complete inhibition was achieved when the DNA molecule was saturated with Ku. This suggested that the inhibitory effect of Ku on LX activity might be due to a restriction of the movement of either Ku or LX along the DNA molecule. In other words, this implied that the ability of Ku to stimulate LX ligation requires movement of one or more molecules along the DNA.

DNA Footprinting Analysis of DNA Molecules with Jointly Bound Ku and DNA Ligase IV·XRCC4—To gain insight into the nature of combined Ku-LX-DNA binding, we next performed footprinting experiments. Because the buffer conditions and protein concentrations required for carrying out the footprinting experiments differed from those used for the end-binding studies in Fig. 4, we first verified that the concentration of Ku used would be non-saturating for binding. The substrate used for these footprinting experiments is 174 bp. At 0.5 and 1.0 pmol of Ku, each DNA molecule has ~1 and 2/3 Ku molecules bound, respectively, similar to the binding pattern observed with the 157-bp fragment (Figs. 5*A* and 4, respectively). LX alone bound to DNA protects ~30 bp at the very end of the molecule (Fig. 5*B*, *lanes 2* and *3*). Ku binds to DNA in a similar way with a slightly shorter footprint covering ~20–25 bp (compare *lanes 3* and *5*). The combined footprint for Ku and LX binding extended to ~55 bp, suggesting that the two bound proteins lie adjacent to one another (Fig. 5*B*, *lanes 7* and *8*). This pattern suggests that either Ku or LX must translocate along the DNA molecule.

Impairment of Ku Translocation by Cisplatin (cis-Pt) Adducts on the DNA Substrate Inhibits the Ku Stimulation of LX Activity—Because the ability of Ku to translocate along DNA molecules has already been well documented (Refs. 17 and 18; reviewed in Ref. 19), we considered it most likely that Ku would be the protein showing inward movement along the DNA molecule. We, therefore, examined whether freedom for Ku to translocate is required for its ability to stimulate LX ligation. To this end, we examined the impact of the presence of *cis*-Pt DNA adducts on ligation, because it had been shown previously that Ku translocation, but not DNA end binding, is inhibited by the presence of *cis*-Pt DNA adducts (24).

A radiolabeled 445-bp *Afl*II-*Pst*I pBluescript fragment either untreated or treated with *cis*-Pt was analyzed in EMSA and ligation assays. *cis*-Pt treatment of the DNA did not significantly affect the binding of Ku to DNA at low concentrations of Ku (0.7 pmol) when only 1 or 2 Ku molecules are bound, demonstrating that *cis*-Pt adducts do not significantly affect Ku end-binding activity (Fig. 6*A*, *lanes 1–4*). At high concentrations of Ku (2.1 and 3.5 pmol) multiple Ku molecules are bound to the DNA substrate as a result of the ability of Ku to translocate and are demonstrated by the multiple band shift products. *cis*-Pt significantly inhibited the formation of these high order complexes consistent with an inhibition of Ku translocation activity (Fig. 6*A*, *lanes 5–8*). The inhibition of Ku translocation is further demonstrated by the use of intermediate Ku concentrations (1.4 pmol) and very short incubation times (Fig. 6*A*, *lanes 9* and *10*). Although multiple Ku molecules are bound to the DNA in the absence of *cis*-Pt, only complexes with 1–3 Ku molecules were detected on *cis*-Pt-treated DNA. These findings are in good agreement with more extensive studies demonstrating that *cis*-Pt adducts inhibit Ku translocation but not Ku binding to DNA ends (24).

Next, the platinized DNA substrate was used for ligation by the LX complex. In the absence of Ku, the ligation efficiency of LX was only slightly lower on the *cis*-Pt-treated DNA compared with the untreated substrate (Fig. 6, *B*, *lanes 1* and *3*, and *C*). Remarkably, the presence of Ku on the *cis*-Pt-treated DNA substrate resulted in severe inhibition of ligation in contrast to the stimulation observed on the undamaged DNA substrate (Fig. 6, *B*, *lanes 2* and *4*, and *C*).

Taken together, our results indicate that the stimulatory effect of Ku on ligation by LX requires freedom for Ku to translocate along the DNA molecule. Inhibition of this ability, either by the presence of multiple Ku molecules or *cis*-Pt adducts, inhibits LX ligation.

Fluorescence Resonance Energy Transfer Studies Show That LX Displaces Ku from the DNA End—The proposal that LX causes inward translocation of Ku differs from a model based on the structural studies, which predicted that the open-ring structure of the Ku·DNA complex would allow free access of proteins at the exposed region of DNA (16). We exploited the technique of FRET, which provides a powerful way to probe spatial dynamics, to examine the interaction between Ku and the DNA end and the impact of LX on this interaction. The oligonucleotide DNA sequence, the donor, was end-labeled with an Alexa Fluor 488 (AF488), and Ku, the acceptor, was labeled

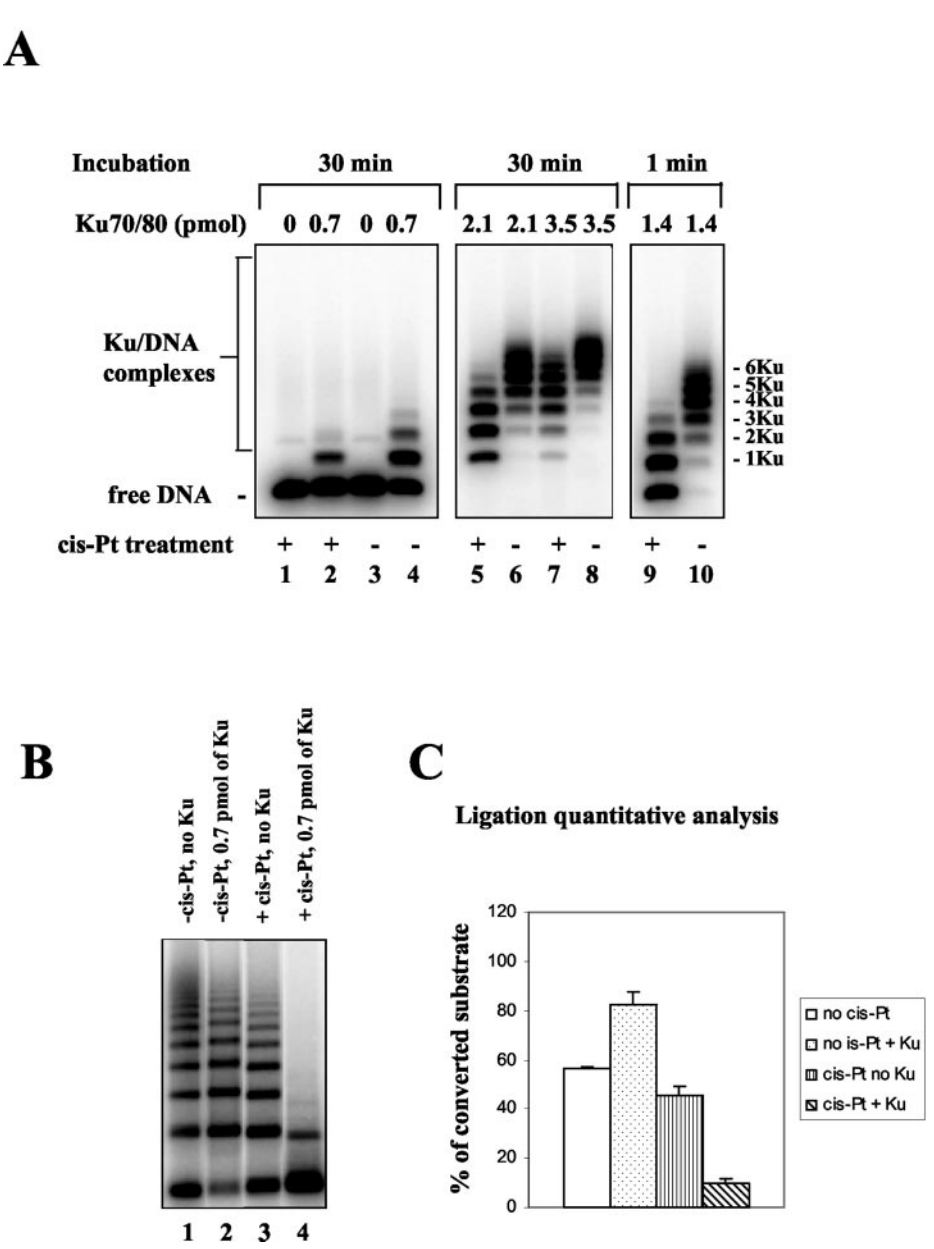

FIG. 6. **Impairment of Ku translocation by *cis*-Pt-induced DNA adducts inhibits ligation activity of LX complex.** *A*, the EMSA analysis of the effect of *cis*-Pt-induced DNA damage on the ability of Ku to interact with the 445-bp *Afl*III-*Pst*I Bluescript fragment. The initial binding of Ku at low concentration (allowing 1 or 2 Ku per DNA molecule) is not impaired by the presence of *cis*-Pt adducts (*left panel*). At higher concentrations, which allow binding of multiple Ku molecules and require Ku translocation, the formation of higher order Ku·DNA complexes is impaired by the presence of *cis*-Pt damage (*middle panel*). Similarly, the impairment of formation of complexes with multiple Ku bound can be detected at intermediate Ku concentration and short incubation times. *B* and *C*, effect of *cis*-Pt-induced DNA damage on the ligation efficiency of LX complex in the presence and absence of Ku (*left panel*). *cis*-Pt treatment of DNA substrate has only slight effect on the ligation efficiency in the absence of Ku (compare *lanes 1* and *3*). In contrast, the presence of 0.7 pmol of Ku stimulates the control reaction, but severely inhibits ligation in the presence of *cis*-Pt adducts (compare *lanes 2* and *4*).

with Alexa Fluor 564 (AF564). We aimed first to show that Ku makes close contact with the DNA end and then to show that LX disrupts that interaction. The molar ratios of Ku:LX:DNA used in these experiments were identical to those used for the footprinting analysis. The DNA-AF488 alone was observed to have a fluorescence lifetime of 3.34 ns, which appeared to be mono-exponential as demonstrated by χ^2 values obtained from mono- and multiexponential analysis (Fig. 7 and Table I). Addition of the acceptor fluorophore (AF564) significantly reduced the average lifetime of the DNA-AF488, indicating the presence of energy transfer. Multiexponential analysis of the data indicates the presence of two species, one likely representing free DNA-AF488 with $\tau \sim 3.34$ and a second interacting fraction with a shorter fluorescence lifetime ($\tau \sim 1.11$) due to the presence of bound Ku making close contact with the DNA end. The relatively high level of DNA without bound Ku is most likely due to the presence of non-fluorescent (unlabeled) Ku. Following preincubation of the DNA substrate with Ku, addition of unlabeled LX complex increased the average fluorescence lifetime to a value close to but not quite reaching the control lifetime (Fig. 7 and Table I). Multiexponential analysis reveals the presence of two fractions with similar lifetimes to

TABLE I

Multiexponential fluorescence lifetime analysis of the FRET data

Summary of fitting parameters determined from the experimental data. t_1 and t_2 are the fluorescence lifetimes, and a_1 and a_2 are the fractions with lifetimes t_1 and t_2, respectively. The reduced goodness-of-fit parameter, χ^2, is used as defined previously (29). An ideal fit would give a χ^2 value of 1. The slightly elevated χ^2 values are due to systematic errors in data acquisition. The restrained fit value shown for DNA-AF488 plus Ku70/80-AF568 plus LX assumes that the FRET coupling between the donor and acceptor remains constant for comparison with the unrestrained fitting (*i.e.* taking the lifetime values from the DNA-AF488 plus Ku70/80-AF568 in the absence of LX). The *first line* of results for each sample is obtained from monoexponential analysis (a single τ value), whereas the *second line* is derived from multiexponential analysis.

	t_1	t_2	a_1	a_2	χ^2
DNA-AF488	3.34		1		1.65
	3.02	3.50	0.35	0.65	1.64
DNA-AF488+Ku70/80-AF568	2.99		1		4.80
	1.106	3.34	0.22	0.78	1.49
DNA-AF488+Ku70/80-AF568+LX	3.21		1		2.08
	1.54	3.72	0.27	0.73	1.19
Restrained fit	1.106	3.34	0.11	0.89	1.37

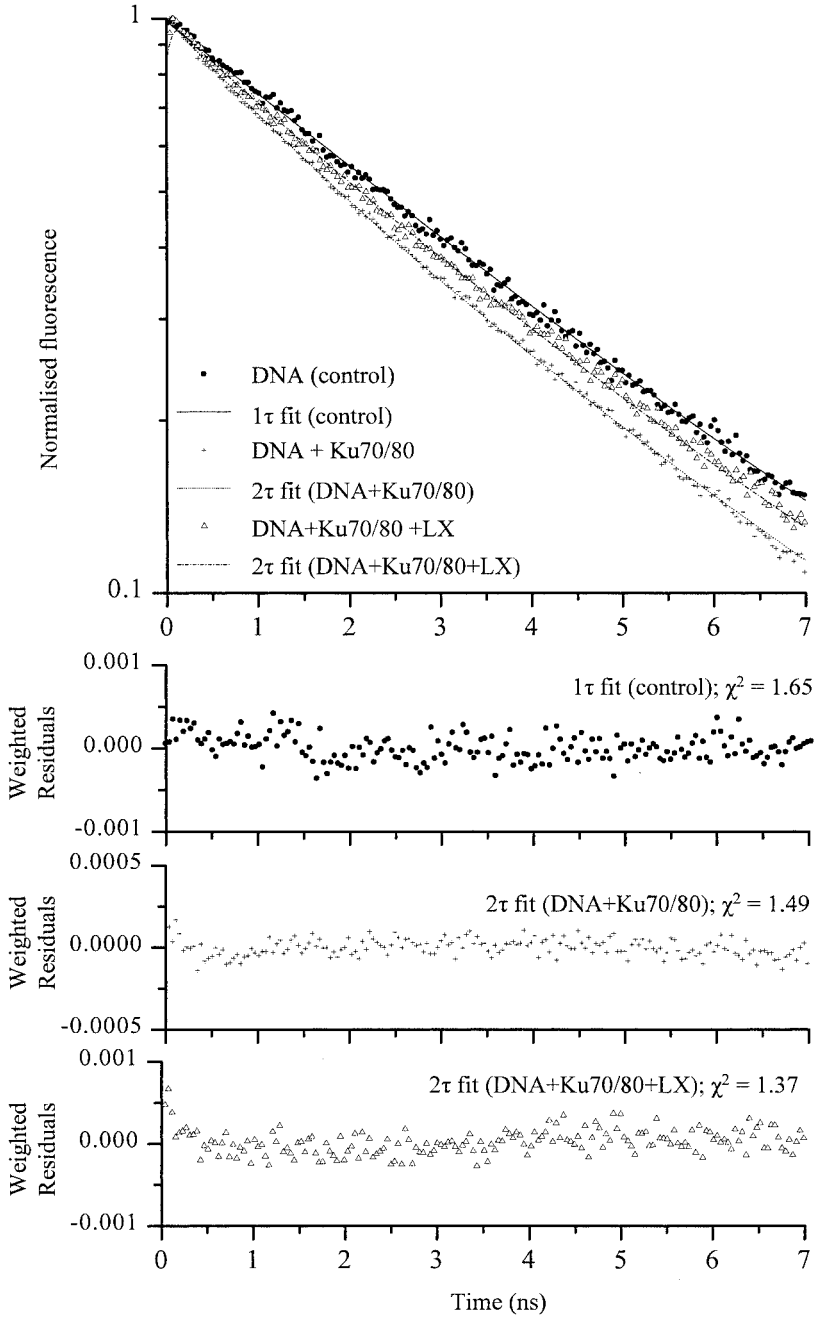

FIG. 7. **Fluorescence resonance energy transfer (FRET) studies show that LX disrupts the contact of Ku with the DNA end.** Normalized fluorescence lifetime data for FRET analysis of interaction between 445-bp DNA-AF488 (donor) and Ku70/80-AF564 (acceptor) in the presence and absence of unlabeled LX complex. The fits are presented with the weighted residuals (as a measure of the suitability of the model) for each data set in addition to the χ^2 goodness-of-fit parameter (see Table I).

those seen in the absence of LX, but the fraction with a reduced fluorescence lifetime due to the presence of Ku ($\tau \sim 1.11$) was reduced. Under these conditions and all others analyzed, we have never observed that LX is able to displace Ku from the DNA (as shown for example by the footprinting and control band shift experiments). Our data, therefore, strongly suggest the fluorophore on Ku makes close spatial contact with the fluorophore on the DNA end and that this close contact is disrupted by the addition of LX. The footprinting experiment suggests that the separation of Ku from the DNA end should increase by 30 bp or 9.96 nm (assuming that each base pair is 0.332 nm). This should lead to an interacting fraction with a lifetime, $\tau_{FRET} \sim 3.33$ ns (with fluorophores ~15.46 nm apart). Such a lifetime would not be distinguishable from the control lifetime. Our findings are therefore compatible with a model showing that the presence of LX at the site of Ku binding disrupts FRET by displacing Ku along the DNA helix. Thus the FRET analysis, taken together with the footprinting analysis, provides strong evidence that Ku is the internally located molecule.

Recruitment of LX by Ku Requires LX Adenylation—Structural studies with smaller DNA ligases have suggested that nick sensing and binding require ligase adenylation, which itself allows an interaction between AMP and the 5′-phosphate at the DNA end (25). We suggest that Ku recruits LX to the DNA end and translocates inwards and that the formation of a stable LX-Ku·DNA complex might require the formation of a DNA·adenylate complex, which itself requires LX adenylation. To examine whether LX adenylation is required for the recruitment of LX to the DNA by Ku, we constructed a mutant DNA ligase IV in which the active site lysine (Lys-273) required for adenylation was mutated to a glycine residue. The K273G mutant protein was co-expressed with wild type XRCC4 in baculovirus as described for the wild type DNA ligase IV and used in band shift studies with substrates of 53 and 157 bp to examine binding to DNA in the presence of Ku. With the 53-bp

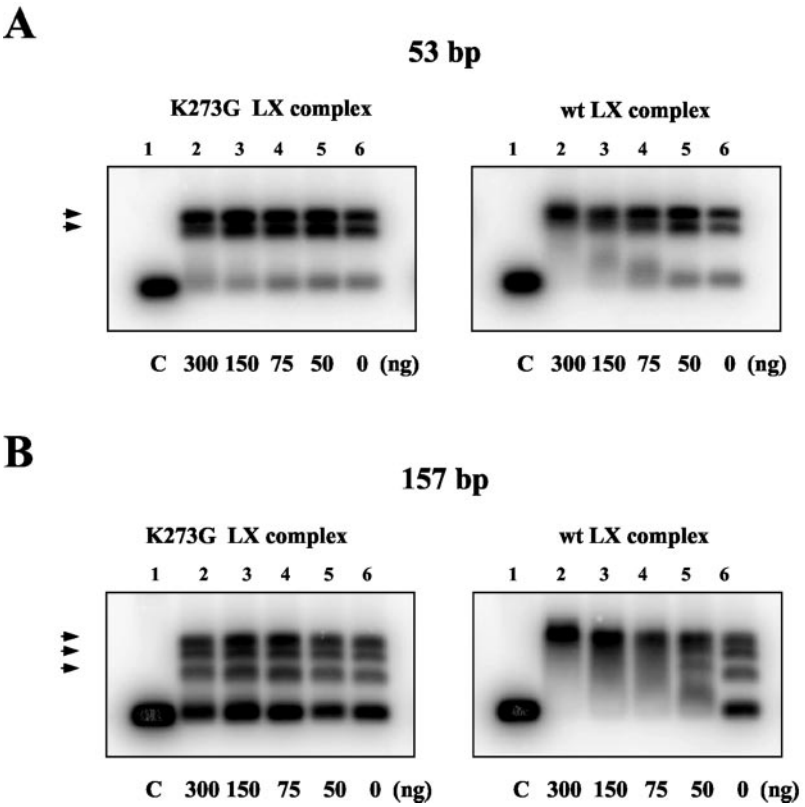

FIG. 8. **Mutation of the adenylation site in DNA ligase IV abolishes the ability of Ku to recruit LX to the DNA.** The K273G mutant DNA ligase IV·XRCC4 complex and a wild type LX complex were employed in band shift experiments using Ku-bound DNA using a 53-bp DNA substrate (*A*) and a 157-bp DNA substrate (*B*). The concentration of Ku was 0.14 pmol, and varying concentrations of LX were employed as indicated.

substrate, the concentration of Ku employed (0.14 pmol) allowed the binding of 1–2 molecules of Ku. At high concentrations of wild type LX, a clear band shift was observed indicative of the formation of a stable LX·Ku·DNA complex (Fig. 8*A*). Such a band shift was not observed with the K273G LX complex. Use of the longer 157-bp substrate demonstrated the same finding more strikingly (Fig. 8*B*). We conclude that the recruitment of LX by Ku requires the adenylation of LX, consistent with the notion that it requires the formation of a DNA·adenylate complex. This finding supports the notion that LX is recruited to the DNA end by Ku rather than being positioned internally on the DNA molecule relative to Ku.

DISCUSSION

We show that LX ligation activity is dependent upon substrate length; although efficient ligation is achieved using a 445-bp substrate, little ligation occurs using a 53-bp substrate. This represents a unique feature of ds ligation catalyzed by LX. The DNA-binding capacity of LX is not influenced by substrate length, and LX can nick ligate a short (53 bp) substrate as efficiently as T4 ligase. Although the binding of the XRCC4 protein has been reported to be more stable on longer DNA molecules, our findings suggest that this is not observed for the intact LX complex (9). This difference may be due to the altered conformation of the coiled-coil motif of XRCC4 in the ternary XRCC4·ligase IV·DNA complex relative to the XRCC4·DNA complex (26). Footprinting shows that LX protects the ends of DNA molecules consistent with a previous finding using atomic force microscopy (15). The substrate length specificity of LX for ligation but not for binding suggests that LX requires access to a larger region of DNA for ligation than is required for binding. It also requires access to a larger region of DNA than is demonstrated by footprinting. ds ligation is likely to require the action of two DNA ligase IV molecules for coordinated ligation of both strands, which may require further movement or repositioning on the DNA. Biochemical evidence indeed suggests that *in vivo* the active LX complex forms a stable mixed tetramer of two DNA ligase IV and two XRCC4 molecules (27).

The stimulation of LX ligation by Ku is independent of substrate length but is critically dependent upon the number of Ku molecules bound to the substrate with pronounced inhibition being observed when multiple Ku molecules are bound. These findings may explain previous reports of stimulation (8) and inhibition of ligation (15) by Ku. It has been shown before that the rate of Ku movement decreases as the number of bound Ku molecules increases (24). Thus, the inhibition of ligation activity of LX at higher concentrations of Ku could be explained by a restriction of the ability of Ku to move along the DNA when there are internal Ku molecules.

Footprinting studies also provided insight into the structure of the Ku·LX complex bound at the DNA ends. The combined Ku·LX footprint is exactly the sum of the two individual footprints and strongly suggests that one or the other molecule moves internally when both are present on the Ku·DNA complex. This was unexpected, because structural studies had suggested that the DNA ends would remain accessible. Although this footprinting analysis does not allow the order of the molecules at the DNA termini to be determined, we considered it likely that Ku is the internally bound molecule considering its known ability to translocate and the fact that LX needs to be in contact with the termini to promote ligation. Three further analyses provide strong evidence that Ku is the internally bound molecule. First, we demonstrate that restriction of the freedom of Ku to translocate due to the presence of *cis*-Pt adducts inhibits LX ligation. LX ligation activity in the absence of Ku was only marginally decreased by the presence of *cis*-Pt adducts at the concentration of *cis*-Pt used, presumably because the random location of the adducts results in only a few molecules with adducts located at their termini. We confirmed previous findings that *cis*-Pt adducts reduce Ku translocation but not its end-binding activity (because few molecules will have adducts at their termini) (24). As predicted, Ku inhibited LX ligation activity on the platinized DNA substrate consistent

with the notion that unrestricted Ku translocation is required for efficient ligation. Second, using FRET analysis, we show that LX can disrupt the contact between Ku and the DNA end. This finding is consistent with the notion that LX becomes the externally bound molecule on the DNA substrate. Finally, this notion is further consolidated by the demonstration that recruitment of LX by Ku requires an adenylated LX complex strongly suggesting that it involves an interaction between LX and the DNA end. Ligase adenylation is a prerequisite for nick sensing by the *Chlorella* ligase (25). A model was proposed by which the ligase molecule moves along the DNA until a nick is encountered. A conformational switch then allows the 3′-OH of the AMP to coordinate directly with the 5′-phosphate of the nick. Our studies suggest that LX does not move along the DNA molecule but rather is recruited by Ku directly to the DNA end where it forms an intermediate involving the AMP moiety on the ligase and the 5′-phosphate at the DNA end. A model whereby LX is recruited directly to the DNA end is also consistent with the fact that *cis*-Pt adducts do not inhibit ligation by LX, suggesting that LX makes direct contact with the DNA end rather than sliding along the DNA in search of the DNA end. Taken together, our results provide strong evidence that the stimulatory effect of Ku on ligation by LX requires the ability of Ku to translocate internally along the DNA molecule. Inhibition of this ability, either by the presence of internal Ku molecules or *cis*-Pt adducts, inhibited LX ligation. It is noteworthy that the translocation of Ku by additional Ku molecules occurs efficiently to generate a DNA molecule saturated with Ku molecules. In contrast, the ability of LX to cause inward translocation of Ku appears to be restricted by the presence of only a few internally bound Ku molecules. Because Ku has an exceptional affinity for DNA ends, this difference is possibly explained by the differing binding potentials of the two molecules and their ability to induce Ku translocation. It is also noteworthy that LX has little ligation activity on the 53-bp substrate, although the footprinting studies indicate that LX only contacts ~30 bp at the DNA termini. This raises the possibility that ds ligation requires movement of LX on the DNA to generate free ends for ligation. This movement may be additional to that required for Ku stimulation of LX activity.

In mammalian cells, NHEJ also requires the DNA-dependent protein kinase catalytic subunit, DNA-PKcs. Our studies have focused on the interactions between LX, Ku, and DNA. However, DNA-PKcs has also been shown to cause inward translocation of Ku (28). In this context, it is important to remember that DNA-PKcs is a late evolutionary addition to the NHEJ machinery and that in the yeast, NHEJ can occur efficiently in the absence of DNA-PKcs. Even in mammalian cells some NHEJ, for example the rejoining of signal junctions during V(D)J recombination, occurs at nearly normal levels in the absence of DNA-PKcs. It is, therefore, possible that DNA-PKcs might have some overlapping function with other NHEJ components and that, in the presence of DNA-PKcs, LX is recruited to a Ku-bound DNA molecule upon which Ku has already translocated inwards. In summary, we propose that Ku, a protein with strong DNA-binding activity, initially recognizes and binds to the short DNA ends generated *in vivo*. Ku binding still allows the recognition of the DNA ends by further Ku molecules or by LX. However, to allow LX binding, Ku translocates internally yielding a complex in which LX is located at the extreme terminus with Ku bound adjacently and internally. This is consistent with the translocation activity of Ku, the ability of LX to disrupt the Ku·DNA end interaction, and the requirement of an adenylated LX complex for recruitment by Ku to the DNA ends. Significantly, restriction of the ability of Ku to translocate inhibits ds ligation. However, although the footprint of the jointly bound LX·Ku DNA molecules covers ~60 bp, it is possible that further inward movement or repositioning of complexes is required for efficient ds ligation.

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