Ku Heterodimer-Independent End Joining in *Trypanosoma brucei* Cell Extracts Relies upon Sequence Microhomology

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DNA double-strand breaks (DSBs) are repaired primarily by two distinct pathways: homologous recombination and nonhomologous end joining (NHEJ). NHEJ has been found in all eukaryotes examined to date and has been described recently for some bacterial species, illustrating its ancestry. *Trypanosoma brucei* is a divergent eukaryotic protist that evades host immunity by antigenic variation, a process in which homologous recombination plays a crucial function. While homologous recombination has been examined in some detail in *T. brucei*, little work has been done to examine what other DSB repair pathways the parasite utilizes. Here we show that *T. brucei* cell extracts support the end joining of linear DNA molecules. These reactions are independent of the Ku heterodimer, indicating that they are distinct from NHEJ, and are guided by sequence microhomology. We also demonstrate bioinformatically that *T. brucei*, in common with other kinetoplastids, does not encode recognizable homologues of DNA ligase IV or XRCC4, suggesting that NHEJ is either absent or mechanistically diverged in these pathogens.

Two principal mechanisms have been described for the repair of DNA double-strand breaks (DSBs). In one pathway, termed homologous recombination, the DSB end(s) invades an intact homologous duplex, allowing DNA resynthesis and religation. Homologous recombination appears to be universal, since its core enzyme is conserved in the three kingdoms of life (31) and in viruses: RecA in bacteria, Rad51 in eukaryotes, RadA in archaea, and UvsX in phage T4 (34). The other pathway, termed nonhomologous end joining (NHEJ), rejoins a DSB directly, without relying on sequence homology. NHEJ is also widely conserved, since the factors involved have been described in all eukaryotes to date (22, 41) and a related process, catalyzed by conserved enzymes, is present in some bacterial lineages (10).

Eukaryotic NHEJ is a multistep reaction catalyzed by a core set of conserved proteins, comprising the Ku70–Ku80 heterodimer (Ku) and a complex of DNA ligase IV (Lig IV; Dnl4 in *Saccharomyces cerevisiae*) and XRCC4 (Lif1 in yeast). Ku binds as a ring to DSB ends, where it can translocate along the duplex (76), and appears to bridge the DNA termini and recruit the other factors for end processing and ligation. DNA Lig IV appears to have evolved specifically for NHEJ, since it interacts with Ku (58) and does not complement the functions of other cellular ligases, which do not function in NHEJ (54, 80). Monomeric DNA Lig IV forms a stable, symmetrical complex with a dimer of XRCC4 (29, 69), which appears to stabilize and activate the ligase and to target it to DSBs. Beyond this core NHEJ machinery, a number of other proteins contribute to the reaction. NEJ1/Lif2 in yeast (33, 74) and XLF/Cernunnos in vertebrates (1, 11) interact with Lig IV–XRCC4 and share sequence similarities with XRCC4 (13), perhaps constituting a protein family whose members are distributed unevenly among eukaryotes (13, 64). In mammals, Ku bound to DNA constitutes two subunits of DNA-dependent protein kinase (DNA-PK), interacting with its catalytic subunit, DNA-PKcs (18). DNA-PKcs appears to aid Ku in protecting and aligning DNA ends, and it interacts with XRCC4 and Artemis, a nuclease that processes DSB ends during V(D)J rearrangements in immune cells (53) as well as some DSBs in other cell types (63). Despite the conservation of DNA-PKcs between vertebrates and some invertebrates (28), the protein is not present in yeast, which may explain the clearer evidence for the MRX11–RAD50–XRS2 (MRX) complex acting during NHEJ in yeast than in mammals. The MRX complex acts in multiple processes during DSB repair and provides at least some of the functions of DNA-PKcs during NHEJ: bridging DNA ends and recruiting the Lig IV–XRCC4 complex (16).

Characterization of NHEJ in bacteria emphasizes that Ku and a specific ligase constitute the core machinery. Ku homologues are found in several bacterial species, frequently in operons with ATP-dependent ligases that are often fused to other functional domains (2, 61). Together, the bacterial Ku and ATP-dependent ligases catalyze a streamlined version of NHEJ compared with that for eukaryotes (24, 36): the bacterial Ku proteins comprise only the eukaryotic Ku “core” domain, they generally function as homodimers, and expression of Ku and ligase from *Mycobacterium tuberculosis* is sufficient to reconstitute functional NHEJ in yeast mutants (24).

Despite the importance of NHEJ in DSB repair, a number of further DNA-joining pathways exist. Single-strand annealing is a pronounced, RAD52-dependent recombination pathway (43, 72) that relies on annealing of direct repeats. Single-strand break repair can also ligate DSBs under some circumstances (3). Finally, a widely observed form of DNA end joining gains prominence in NHEJ mutants and is mechanistically distinct in that significant DNA deletions occur around...
the DSB, and joining relies on stretches of microhomology notably longer than those in Ku-dependent reactions (9, 38, 45, 51, 70, 75, 81). A number of in vitro studies have also examined these DNA end-joining reactions. NHEJ has been reconstituted using purified components from mammals (52) and yeast (16). More-extensive studies, to date limited to mammalian cells and Xenopus laevis, have examined DNA end joining in cell and nuclear extracts (47), where Ku-dependent NHEJ (4, 12, 14, 17, 42, 46), as well as the other forms of end joining observed in vivo (14, 17, 32, 46, 50, 60, 67, 73), can be seen.

In this study, we have examined DNA end joining in Trypanosoma brucei, a divergent eukaryotic parasite (40) that is the causative agent of sleeping sickness in humans and nagana in cattle. T. brucei survives in mammals due to antigenic variation, a process that involves switching of the variant surface glycoprotein (VSG) coat. The T. brucei genome contains >1,000 VSG genes, found predominantly in silent arrays (6), and switching occurs mainly by recombination of VSG gene copies from the silent loci to telomeric sites of transcription. Antigenic variation can occur at rates much higher than that of background mutation, and genetic evidence implicates homologous recombination in at least some of the VSG switching reactions (56, 62).

Little is known about other DSB repair pathways in T. brucei. Although KU70 and KU80 are present in T. brucei and function in telomere maintenance (19, 44), a role conserved in other eukaryotes, no evidence has been provided to suggest that NHEJ occurs in vivo, despite a number of attempts to assay the reaction (19, 20). Here we looked for Ku-dependent NHEJ by assaying for DNA end joining in T. brucei cell extracts. However, only microhomology-based reactions that are independent of Ku and are highly reminiscent of a reaction pathway described during T. brucei transformation (20) were observed. Using bioinformatic analyses, we find that DNA Lig IV and XRCC4 may not be encoded by the genome of T. brucei and related kinetoplastids, raising the possibility that NHEJ either is absent or utilizes diverged ligase factors.

**MATERIALS AND METHODS**

*T. brucei* growth and cell extract preparation. *T. brucei* procyclic-form cells of strain EATRO705 were grown in SDM-79 medium at 27°C. Approximately 1 liter of cells at densities between 0.8 × 10^9 and 2.0 × 10^9 cells ml⁻¹ was used to prepare a nuclear or cell extract. Bloodstream-stage cells of strain Lister 427 or ILTat1.2 were grown in HMI-9 medium at 37°C and used to infect adult female ICR mice (Harlan, United Kingdom) that had been immunocompromised with cyclophosphamide (250 mg kg⁻¹ of body weight). At a parasitemia of 0.5 × 10^9 to 1.0 × 10^9 cells ml⁻¹, the mice were sacrificed, and 0.5 ml of blood was used to infect adult female Wistar rats (immunocompromised as described above; Harlan, United Kingdom). When the parasitemia here reached 0.5 × 10^9 to 1.0 × 10^9 cells ml⁻¹, ~10 ml of blood was withdrawn by cardiac puncture into Carter’s balanced salt solution containing 5% sodium citrate anticoagulant, and T. brucei was then purified by DE52 anion-exchange chromatography. Nuclear extracts were prepared in glycero/cytosol-containing buffers as described previously (5). Whole-cell extracts were obtained by the procedure of Laufer et al. (48), using sucrose-containing buffers. RAD51 and KU70 homologous mutants were generated in procyclic-form *T. brucei* EATRO705 by using constructs and transformation conditions described previously (19, 56).

**End joining.** Standard reactions proceeded for 10 min at 37°C and used 10 to 20 μg of cell extract and 200 to 500 ng of DNA in a total volume of 100 μl containing 50 mM Tris−HCl (pH 7.5), 20 mM potassium acetate, 3 mM magnesium acetate, 1 mM ATP, 1 mM dithiothreitol, and 100 μg ml⁻¹ bovine serum albumin. A 5-min preincubation of the extract preceded addition of the DNA. For reactions involving ATP regeneration, 10 mM creatine phosphate and 20 μg ml⁻¹ creatine kinase were included (both from Roche). To deplete ATP, 10 U of apyrase (New England Biolabs) was added to each reaction mixture, and the extract was incubated for 10 min at 37°C prior to substrate addition. Reaction products were prepared for analysis by phenol-chloroform extraction and ethanol precipitation and were normally examined by Southern blotting and hybridization with [α-³²P]-labeled substrate DNA. For experiments using whole-cell extracts, the products were treated with 0.2 mg ml⁻¹ RNase A (Sigma) for 2 min prior to phenol-chloroform extraction. Hybridization was visualized using a PhosphorImager (Typhoon 8610, Molecular Dynamics) and quantified by densitometric analysis using ImageQuant.

pBluescript (Stratagene) and other plasmids were prepared for end joining by digestion of ~20 μg of DNA with the appropriate restriction enzyme (see below) at 37°C for 2 h and were then purified by phenol-chloroform extraction and ethanol precipitation; the concentration and extent of digestion were analyzed by agarose gel electrophoresis prior to end joining. PCR products to assay end joining were amplified from the NEO or HYG gene in *T. brucei* strain 3174 (56) genomic DNA by using Taq polymerase (ABgene) and oligonucleotide primers that had been gel purified; after PCR, the products were purified by phenol-chloroform extraction and ethanol precipitation or by spin purification (Qiagen) and were assessed for specificity and quantity by agarose gel electrophoresis. Joints were analyzed by purifying the end-joining reaction products as described above, separating half the reaction product by agarose gel electrophoresis, and performing PCR amplification on the other half with Taq polymerase. The resultant PCR products were TOPO cloned (Invitrogen), multiple clones were isolated, and products of representative sizes were sequenced. Control reactions were performed with the HYG joint primers on the end-joined NEO substrate, with NEO joint primers on the end-joined HYG substrate, and with both primer pairs on substrates that had not been incubated in the nuclear extract; the reaction products were analyzed by gel electrophoresis and were TOPO cloned and sequenced, showing either no amplification or small amounts of a single-primer product. All oligonucleotide primers used for this work can be provided on request.

**Bioinformatic analysis of NHEJ factors.** To search for homologues of the core NHEJ components in the kinetoplastids, polypeptide sequences for Ku (the 70 and 80 subunits), DNA ligase (I, III, or IV), or XRCC4/Lif4 from a number of species were identified from annotations within the NCBI database and used initially to search the *T. brucei*, Trypanosoma cruzi, and Leishmania major genome project databases by BlastP (default settings). Iterative searches were also used to attempt to identify remote homologues. To ensure that the searches were sensitive, and to attempt to exclude false positives, bespoke hmmer profiles were created from multiple sequence alignments of the different protein sequences by the Hmmbuild and hmmbuild2 programs, and conceptual translations from individual genome projects were analyzed using the hmmssearch program. The NR database was mined for homologues with the bespoke profiles using the Kyoto University Bioinformatics Center MOTIF service (http://motif.genome.jp), demonstrating the validity of the profiles. All potential hits were checked manually against known sequence motifs and domain structures.

For phylogenetic analyses, multiple alignments of the different protein sequence alignments were performed using each of the following methods: ClustalW (default settings), Hmmalign (displaying match states to the profile only), Matchbox (default parameters) (25), T-coffee (default parameters) (59), and Fugue (68) alignment against a pdb structural file or the Homstrad profile appropriate to the case. Where subsequences of known domains were required, sequences were aligned against the appropriate profile from the Pfam database using the hmmbuild program. The leading and trailing portions of the alignment outside the match to the profile were then removed and the sequences converted to fasta format for subsequent analysis. The alignments made using the different methods were then combined by the T-coffee COMBINE function. For the ATP-dependent DNA ligases, the consensus alignment was written in phylip format, and a phylogenetic tree was generated by the Phylip package utilizing protdist and fitch. The significance of the different DNA ligase groupings was determined by bootstrap analysis of 100 replicates, and each had a >90% probability (data not shown).

**RESULTS**

*T. brucei* nuclear extracts catalyze efficient joining of linear DNA molecules. To look for end-joining activity in *T. brucei*, we examined the ability of nuclear extracts to catalyze the joining of linear DNA molecules. First, SacI-digested pBluescript DNA was incubated with an extract from ILTat1.2 bloodstream-stage cells (Fig. 1A). DNA joining was detected readily, resulting in dimer- and trimer-sized linear molecules,
as well as higher forms that appear to result from the substrate joining to genomic DNA (the molecules are >12 kb). All the reaction products were joined by covalent linkages, as evidenced by the fact that their electrophoretic mobilities were unaffected by heating of the reaction products to 90°C (data not shown). To examine the efficiency of the reactions, time courses were then performed (an example is shown in Fig. 1B), revealing that DNA joining was rapid, with dimer-sized products appearing after 30 s. Quantification of a number of independent experiments using distinct plasmids (Fig. 1C) showed that the dimer product became most abundant after 5 to 10 min and diminished thereafter. Most likely, this is due to nuclease digestion by the extract, since the decrease in the amount of the substrate over time (only around 50% remained after ~20 min) was greater than could be explained by conversion to joined products. In all DNA-joining reactions, using nuclear extracts and plasmids or smaller PCR products (see below) as substrates, we observed intermolecular joining; only in whole-cell extracts (see below) did we see any evidence for intramolecular joining.

To determine if end joining is strain or life cycle stage specific, the reactions in nuclear extracts from Lister 427 bloodstream-stage cells (+) and from EATRO795 procyclic-form cells were compared. Equal efficiency was found in each (data not shown). We next attempted to optimize the reaction conditions (data not shown). A linear increase in the quantity of the reaction product was seen as the amount of the extract was increased over the range 5 to 100 µg. The reaction occurred with optimal efficiency between 35 and 37°C (becoming less efficient at higher and lower temperatures over the range 25 to 45°C), and maximum joining was found between 20 and 40 mM potassium diacetate and was undetectable above 100 mM.

To assess whether ATP and magnesium are required for DNA joining, a 798-bp DNA fragment was PCR amplified using 5'-phosphorylated oligonucleotide primers. DNA joining was not observed if Mg2+ was omitted from the reaction mixture (Fig. 2A). Moreover, joining of plasmid DNA occurred efficiently up to 3 mM Mg2+ but became less efficient at higher concentrations (Fig. 2B). Since the substrate appeared to be degraded more rapidly at higher Mg2+ concentrations, the reduced end joining may be due to increased nucleolytic digestion. Surprisingly, end joining was equally efficient in nuclear extracts whether exogenous ATP (1 mM) was added or omitted (Fig. 2A). Although we cannot rule out the possibility that sufficient ATP for the reaction is present in the nuclear extract, this seems unlikely; T. brucei contains 22 nmol of ATP/mg of protein (21), meaning that maximally 4.4 µM ATP could be present in an unsupplemented reaction mixture using 20 µg of extract (assuming, erroneously, that ATP is not depleted during extract preparation). In fact, T. brucei contains significant glycerol kinase activity (39), which would deplete the nuclear extract of ATP due to the use of glycerol in the preparation buffers (A. Gunzl, personal communication). We therefore generated whole-cell extracts that were prepared in a sucrose-containing buffer (and should retain ATP) and compared joining on a linear pBluescript plasmid with or without added ATP (1 mM) or with addition of an ATP regeneration system (Fig. 3A). Overall, end joining was slightly less efficient in whole-cell extracts than in nuclear extracts. In addition, the product profile in whole-cell extracts was more variable and
somewhat distinct from that in nuclear extracts: although dimer-sized products were still detectable, other molecules, both larger and smaller than the substrate, were generated in some reactions. It is possible that these products result from intramolecular joining, or potentially from truncation of the substrate, but we have not verified this, because such products were not generated uniformly in every experiment (e.g., compare Fig. 3A and B). Nevertheless, exogenous ATP did not increase end joining substantially, as determined by the amounts of the product generated over time. Addition of an ATP regeneration system also did not increase the amount of the product, nor did it increase the longevity of the reaction. In fact, the substrate was depleted more rapidly under these conditions. To examine this further, we asked if depletion of putative ATP in the extract by the addition of apyrase would affect the reaction. Again, we found no evidence that the end-joining activity was reduced by this treatment (Fig. 3B).

**T. brucei DNA joining is mediated by microhomology.** To determine if the end-joining activity occurs precisely, as has been described for error-free Ku-dependent NHEJ reactions (4), or involves sequence changes indicative of error-prone Ku-dependent NHEJ (12) or Ku-independent joining (50), further substrates were derived by PCR. In one PCR, a 400-bp DNA fragment was amplified with primers containing terminal recognition sites for Acc65I, KpnI, or EcoRV, which create termini with a 5' overhang, a 3' overhang, or a blunt end, respectively. The different restriction-digested substrates were incubated with a *T. brucei* nuclear extract under “standard” conditions (see Materials and Methods). An example of this analysis is shown in Fig. 4A, indicating that the DNA end configuration did not influence the efficiency of joining. To ensure that this finding was not due to inefficient restriction digestion of the PCR products, we compared the end-joining efficiency of plasmid DNA that had been similarly restriction digested with the nuclear extract, where 3' terminal A overhangs were joined most efficiently, 3' overhangs are joined slightly less efficiently, and blunt ends are the poorest substrates (4, 12). In fact, in *T. brucei* nuclear extracts, the same PCR product that had not been restriction digested was joined as efficiently as the other three substrates (Fig. 4A). This indicates strongly that the DNA end configuration has little influence on end joining, since such *Taq*-generated PCR products lack 5’-terminal phosphates and contain 3’-phosphorylated A overhangs, both of which are conditions refractory to ligation and NHEJ (23). To examine this further, a 391-bp substrate was generated by PCR with non-phosphorylated primers, each containing a terminal HindIII site. HindIII-digested or undigested substrates were then incubated with the nuclear extract, the reaction products were deproteinized, and half of each was redigested with HindIII. The majority of the products could not be digested with HindIII (Fig. 4B), showing that *T. brucei* DNA end joining is predominantly error
prone, removing terminal sequences irrespective of whether complementary overhangs are present.

To determine how DNA joining occurs in *T. brucei* extracts, a 400-bp 5′-nonphosphorylated substrate (NEO) and a 798-bp 5′-phosphorylated substrate (HYG) were incubated in separate, standard reactions using either nuclear or whole-cell extracts. In each case, half of the reaction product was analyzed by gel electrophoresis to confirm joining (data not shown). To characterize the joins, PCR was performed on the other halves of the reaction products using primers internal to the substrates, which should amplify across the joins of head-tail, head-head, or tail-tail product configurations. For both substrates, several different-sized DNA fragments were PCR amplified (data not shown), and cloning and sequencing revealed that these were derived only from head-tail joins (Fig. 5). Most likely, the other joining configurations occur, but PCR is impeded by the formation of hairpins during the annealing of the PCR products. In control experiments where the substrates had not been incubated with an extract, very limited PCR amplification occurred by fortuitous, single-primer amplifications (data not shown).

All intermolecular joins occurred at regions of microhomology within the DNA substrates, irrespective of whether the reaction had been conducted in nuclear or whole-cell extracts. These regions ranged from 6 to 16 bp and virtually always (7 of 8 joins) contained at least 1 mismatched base. Mismatch repair does not appear to occur during joining, however; the sequences of the microhomologous joins were derived from one of the other substrate molecule and were not hybrids (data not shown). In creating the joins, sequence was almost always lost during the formation of hairpins during the annealing of the PCR products. In control experiments where the substrates had not been incubated with an extract, very limited PCR amplification occurred by fortuitous, single-primer amplifications (data not shown).

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predicted kinetoplastid DNA ligases, and searches of the kinetoplastid genomes using these sequence elements in isolation revealed no other putative DNA ligases. Taking these data together, it is likely that we have identified all the ATP-dependent ligases encoded by these three kinetoplastids, and none of those present are recognizably related to DNA Lig IV.

Searches for XRCC4/Lif1 are more problematic, because the primary sequence of this protein is poorly conserved in eukaryotes (29, 69). Perhaps not surprisingly, initial BlastP searches of the kinetoplastid genomes failed to indicate any XRCC4/Lif1 homologues. To examine this further, we generated an hmm profile from the multiple alignment of the DNA Lig IV interacting domain published by Sibanda et al. (69). Searches using this hmm profile also failed to identify any protein in the kinetoplastid genomes. Although the NHEJ factors beyond the core machinery are less widely conserved, we also searched the kinetoplastid genomes for each of these proteins (DNA-PKcs, Artemis, NEJ1, XLF), and no detectable homologue was found (data not shown).

DISCUSSION

To our knowledge, these experiments represent the first description of DNA end joining catalyzed by cell extracts of any single-cell organism. Despite the presence of both subunits of the Ku heterodimer in *T. brucei*, where Ku functions in telomere maintenance (19, 44), the joining activity we have observed is not NHEJ. The reactions characterized occur efficiently in nuclear extracts of *T. brucei ku70* mutants and rely on regions of microhomology. These are characteristics of microhomology-mediated end joining (MMEJ), which is nor-

FIG. 5. Sequences of joints during *T. brucei* end joining. DNA sequences of head-tail-ligated products of NEO (N) or HYG (H) PCR substrates following end joining in *T. brucei* cell extracts were determined following PCR amplification using primers internal to the substrates. For each product, the top sequence depicts the upper (head) strand (5'-to-3' orientation) of one molecule where it has been ligated to another (tail) molecule (bottom sequence, same orientation); the joints formed between the molecules were, in each case, within regions of sequence homology (underlined) revealed by nucleolytic degradation of the DNA ends (the numbers of bases deleted [Δ] are given). The relative abundance of each product is shown on the right, expressed as the number of clones isolated with a given joint sequence (from a total of 27) and the percentage of the total that this represents.

FIG. 6. *T. brucei* end joining does not require RAD51 or KU70. The efficiency of end joining in nuclear extracts of wild-type procyclic-form *T. brucei* was compared to that in extracts from homzygous mutants (−/−) of RAD51 or KU70. Increasing amounts of the extract from each cell line, compared with control reactions where no extract was added (−), were used. The substrate monomer (M) and dimer (D)-sized molecules are indicated.
activity in other in vitro assays appears to be sensitive to substrate and extract concentrations, with Ku-dependent NHEJ predominating at high protein concentrations (>0.5 to 2 μg · μl⁻¹) relative to DNA (<5 ng · μl⁻¹) (4, 50); in fact, high protein concentrations (>1 to 2 μg · μl⁻¹) eventually inhibit detectable joining (8, 50). Over the range of 0.25 to 1 μg of extract · μl⁻¹, using 1 ng of DNA · μl⁻¹, we find a linear increase in the amount of the reaction product (data not shown) and have only ever observed microhomology-mediated, presumably Ku-independent, joining. To determine whether or not Ku-dependent NHEJ occurs in *T. brucei* extracts requires further work, but the predominance of MMEJ appears reminiscent of end-joining reactions seen in other euukaryotes when Ku-dependent NHEJ is absent, by investigators either examining end joining in core NHEJ mutants using cell extracts (14, 17, 26, 32, 50, 77) or in vivo assays (45, 70, 75), immunodepleting extracts of core NHEJ proteins (4, 14, 46, 67, 77), inhibiting DNA-PK with wortmannin (4, 60), or performing extract fractionation (37, 55).

The *T. brucei* end joining we have described in vitro is highly reminiscent of DNA recombination reactions observed in vivo during experiments to integrate plasmid constructs into the *T. brucei* genome following transformation (20). The in vivo and in vitro reactions are each RAD51 independent and rely on stretches of DNA microhomology with very similar sequence characteristics. Moreover, the in vivo integration reactions frequently result in visible reductions in the size of the targeted chromosome, perhaps suggesting that they arise from end joining of the plasmid to endogenous DSBs. It seems plausible, therefore, that the Ku independence of the joining we have described in vitro suggests that the in vivo reactions are also Ku independent MMEJ. The function(s) of the MMEJ reaction in *T. brucei* is unknown. It seems likely that the reaction provides a means for repairing DNA damage, though it may also serve other functions. Irrespective of this, the fact that MMEJ is detectable in a diverged protist such as *T. brucei* suggests that it is a conserved repair pathway. Moreover, if NHEJ is absent from *T. brucei* (discussed below), this may be an attractive organism with which to perform this characterization. As a caveat, we note the perplexing finding that we cannot provide evidence that *T. brucei* MMEJ in vitro requires ATP, an expectation for ATP DNA ligase-dependent reactions in other organisms. Identification of the factors involved in *T. brucei* MMEJ would allow us to address all the above questions. However, this may not be straightforward, since the enzymatic machinery involved in MMEJ in other euukaryotes remains unclear (51, 78).

To attempt to understand why NHEJ has not been detected in *T. brucei* or related kinetoplastids despite extensive genetic manipulation over many years (7), we used bioinformatic search tools to look for core NHEJ factors encoded by kinetoplastid genomes. Despite extensive searches and the clear presence of each component of the Ku heterodimer, we have not been able to identify either DNA Lig IV or XRCC4/Lif1, the key ligation components of NHEJ. Further experiments will be needed to investigate whether this means that NHEJ is absent in these organisms or whether the reaction utilizes a ligase distinct from DNA Lig IV. Nevertheless, in vivo work with *T. brucei* suggests that NHEJ, if it occurs at all, does not operate at significant levels. During extensive genetic manipulation of
The Kinetoplastida may suggest that the protein heterodimer mutagenic process than NHEJ. In addition, retention of Ku in significant loss and changes in DNA sequence appear to be as-
cocations for genome structure and function. For instance, sig-
kingdom is unclear. An absence of NHEJ would have impli-
(35). Moreover, a Ku homologue has been described for only
matic machinery may be absent from
it is accepted that the reaction is absent from many bacterial
understanding of the evolution of this repair reaction. Though
karyotes is worth pursuing. Such a finding would alter our
Ku plays only a minor role in DNA repair.
Finally,
chromosomal rearrangements, no such translocations are ob-
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