3348–3354 *Nucleic Acids Research, 1999, Vol. 27, No. 16*

Mammalian mitochondrial extracts possess DNA end-binding activity

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Received March 24, 1999; Revised and Accepted June 21, 1999

ABSTRACT

Mammalian mitochondrial protein extracts possess DNA end-binding (DEB) activity. Protein binding to a 394 bp double-stranded DNA molecule was measured using an electrophoretic mobility shift assay. Mitochondrial DEB activity was highly specific for linear DNA. Inclusion of a vast excess of non-radioactive circular DNA did not disrupt binding to radioactive f394. In contrast, binding was abolished by the inclusion of linear competitor DNA. In mammals, nuclear DEB activity is due to Ku, a heterodimer composed of the Ku70 and Ku86 proteins. To determine whether mitochondrial DEB activity was also due to Ku, protein extracts were prepared from the Chinese hamster XR-V15B cell line, which lacks this protein. As anticipated, nuclear extracts prepared from these cells lacked DEB activity. In contrast, mitochondrial extracts prepared from these cells had wild-type levels of DEB activity, demonstrating that this latter activity is not a consequence of nuclear contamination. Although the nuclear and mitochondrial DEB activities are independent of each other, they are nevertheless closely related, since mitochondrial DEB activity was 'supershifted' by both anti-Ku70 and anti-Ku86 antisera. The nuclear DEB protein Ku plays an essential role in nuclear DNA double-strand break repair. The DEB activity described herein may therefore play a similar role in mitochondrial DNA repair.

INTRODUCTION

Mammals possess two cellular genomes, one in the nucleus and a second within the mitochondria (1). Nearly 25 years ago it was shown that, in contrast to the nucleus, mammalian mitochondria are unable to repair UV photodamage (2). This observation, which has recently been confirmed (3,4), formed the cornerstone of the hypothesis that mammalian mitochondria are devoid of DNA repair activity (5). According to this theory, the high cellular copy number of the mitochondrial genome rendered individual mitochondrial DNA (mtDNA) molecules functionally redundant, thereby obviating the need for DNA repair within this organelle.

However, a number of findings strongly refute this notion. In particular, there is compelling evidence that a mitochondrial base excision repair pathway exists. First, a mitochondrial version of the uracil DNA glycosylase enzyme has been identified (6). The mitochondria-specific form has been shown to result from alternative splicing of the gene encoding the nuclear version of this enzyme, which functions to remove uracil residues misincorporated into DNA during replication (7). An alternate form of DNA ligase III is similarly targeted to the mitochondria (8). Second, purified *Xenopus laevis* mitochondrial enzymes can efficiently repair abasic sites in DNA *in vitro* (9). Third, mitochondrial forms of oxidative damage-specific endonuclease (10) and apurinic/apyrimidinic nuclease (11) have been identified. Fourth, studies have detected the repair of oxidatively damaged mitochondrial DNA in rat (12) and Chinese hamster ovarian (13) cells. Taken together, these results strongly argue for the presence within mammalian mitochondria of a base excision DNA repair pathway that closely resembles that found in the nucleus of these cells.

These experiments prompted an investigation into other nuclear DNA repair pathways that may also function in mammalian mitochondria. Results from such studies indicate that common pathways to repair damaged DNA exist in both organelles. For example, it is known that mammalian nuclei rely on both homologous recombination and non-homologous end-joining to repair DNA double-strand breaks (reviewed in 14). Similarly, mammalian mitochondrial protein extracts possess both homologous recombination and non-homologous DNA end-joining activity (15,16). The existence of these activities in mitochondrial protein extracts is consistent with the hypothesis that pathways for the repair of DNA double-strand breaks function in that organelle.

Efficient nuclear non-homologous DNA end-joining is dependent upon Ku, a heterodimeric protein comprised of two subunits, Ku70 and Ku86 (reviewed in 17). Cells deficient in either of these proteins display hypersensitivity to X-rays (18–22). In addition, nuclear extracts prepared from these mutant cells lack a characteristic DEB activity that is detected using an electrophoretic mobility shift assay (20,23). Studies with purified protein have proved that Ku binds with high affinity to the ends of linear DNA molecules (24). Furthermore, a recent report has shown that addition of Ku protein substantially enhances the ability of purified mammalian DNA ligase to re-join linear DNA substrates *in vitro* (25). A series of recent papers have identified yeast homologs of Ku70 and Ku86 and shown that these proteins are essential for non-homologous end-joining in that organism (19,20,26–28).

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We therefore reasoned that if mammalian mitochondria possess a non-homologous DNA end-joining pathway, extracts prepared from this source would likely contain a DNA end-binding (DEB) activity functionally equivalent to Ku. Consistent with this hypothesis, we detected DEB activity in mammalian mitochondrial extracts. Mitochondrial DEB activity in cells lacking a functional Ku86 gene is identical to that present in wild-type cells. This indicates that DEB activity in mitochondrial protein extracts is not due to contamination by nuclear proteins. The possible role this DEB activity may play in the mitochondria is discussed.

MATERIALS AND METHODS

Cells

Chinese hamster cell lines V79 and XR-V15B and human HT1080 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, sodium pyruvate, penicillin, streptomycin and amphotericin B. Livers were obtained from adult Fisher 344 rats.

Isolation of mitochondria

Mitochondria from mammalian cells or rat liver were prepared as described (29). Briefly, cells were washed with phosphate-buffered saline (PBS) and sedimented by centrifugation at 1300 *g* for 5 min. Cells were then resuspended in a buffer containing 0.3 M sucrose, 1 mM EGTA, 5 mM MOPS, 5 mM KH_2PO_4, 0.1% BSA, pH 7.4, and disrupted using a Dounce homogenizer (three consecutive sets of 20 strokes). The treated cells were centrifuged three times at 2600 *g* for 5 min. The resulting supernatant was centrifuged at 15 000 *g* for 10 min to obtain a crude mitochondrial fraction. This mitochondrial fraction was purified by Percoll density gradient centrifugation. The integrity of the mitochondrial preparation was assessed by measuring cytochrome c oxidase activity (30).

Isolation of nuclei

Cells were rinsed with PBS, scraped from tissue culture dishes and collected into centrifuge tubes, then sedimented at 1000 *g* for 5 min. Cells were then resuspended in a buffer containing 10 mM KCl, 10 mM $MgCl_2$, 10 mM Tris, pH 7.4, and 10 mM DTT and homogenized. Disrupted cells were centrifuged three times at 1500 *g* for 5 min to separate the nuclei from the supernatant.

Preparation of protein extracts

Protein extracts were prepared by resuspending intact mitochondria or nuclei in a buffer containing 10 mM KCl, 10 mM $MgCl_2$, 10 mM Tris, pH 7.4, 10 mM DTT and 350 mM NaCl in the presence of pepstatin (0.7 μg/ml), aprotinin (1 μg/ml), leupeptin (0.2 μg/ml) and PMSF (1 mM). Preparations were incubated for 1 h on ice. Lysates were then centrifuged for 30 min in a Beckman Ultra-TL100 centrifuge at 70 000 r.p.m. The supernatant was dialyzed against 25 mM Tris, pH 7.5, 1 mM EDTA, 1 mM PMSF, 1 mM DTT and 10% glycerol, concentrated and stored at –80°C. Protein concentration was determined using the Bradford assay (31).

Preparation of DNA substrates

The DNA fragment f394 is a 394 bp restriction fragment (spanning nt 2358–2752) released from the plasmid pREP4 (Invitrogen) upon digestion with *Eco*RI. The fragment was gel purified, then end-labeled using the Klenow large fragment of *Escherichia coli* DNA polymerase I (New England Biolabs) and [α-^{32}P]dATP.

Circular ϕX174 replicative form DNA was used as circular competitor DNA for electrophoretic mobility shift assays. Linear competitor DNA was prepared by restriction endonuclease digestion of replicative form ϕX174 with *Ase*I and *Mlu*I or $pREP_4$ with *Dpn*I, as indicated for each experiment. Competitor DNA substrates with different ends (i.e. a 5'-overhang, a 3'-overhang or blunt-ended) were generated by cutting ϕX174 with *Ava*I, *Pst*I or *Stu*I, respectively.

Electrophoretic mobility shift assay (EMSA)

A modification of the assay used by Rathmell and Chu (23) was employed to detect DEB activity in mitochondrial and nuclear extracts. The binding reaction contained radioactive f394 probe, unlabeled supercoiled plasmid DNA (to compete for non-specific DNA-binding proteins), unlabeled linear plasmid DNA (to specifically compete for DEB activity) and mitochondrial or nuclear extract in binding buffer (12 mM HEPES, 5 mM $MgCl_2$, 4 mM Tris, pH 7.9, 100 mM KCl, 0.6 mM EDTA, 0.6 mM DTT and 12% glycerol). The amount of linear and circular competitor DNA as well as the amount of radioactive DNA included in the reaction is indicated for each experiment. The final volume of the reaction was 10 μl. The appropriate amount of protein extract was added to initiate the assay and the reaction was incubated at 14°C for 30 min. Following incubation, 5× loading buffer (0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol) was added to each reaction and the entire reaction was subjected to electrophoresis on a 5% polyacrylamide gel in 1× TBE (90 mM Tris–borate, 2 mM EDTA) running buffer. The gel was run at 20 V/cm for ~2 h at 4°C, then dried. Detection of radioactivity was achieved using a phosphorimager (Molecular Dynamics).

Supershift assay

An EMSA (see above) was conducted. Following the 30 min incubation period at 14°C, between 10 and 16 μg of anti-Ku70 or anti-Ku86 polyclonal antiserum (provided by Drs Susan Critchlow and Stephen Jackson, Wellcome/CRC Institute, UK) or rabbit IgG antibody (Sigma) was added to the reaction. Antibody was incubated with the binding reaction at 4°C for 1 h. Loading buffer (5×) was then added to each reaction and the entire contents loaded onto a 5% polyacrylamide gel in TBE running buffer. Gel electrophoresis and radioactivity detection were carried out as described above.

Western blot analysis

Aliquots of 5 μg of either nuclear or mitochondrial protein extract were fractionated by 10% SDS–PAGE, then electrophoretically transferred for 1 h to a nitrocellulose membrane. The membrane was submersed in 5% BSA in PBS for at least 1 h. Membranes were then probed with either anti-Ku70 or anti-Ku86 polyclonal antiserum (1/2500 dilution in 5% BSA in PBS) for 1 h with gentle agitation, then washed for 15 min in three changes of 0.02% BSA in PBS. Following this treatment, membranes were probed with alkaline phosphatase-conjugated, goat anti-rabbit IgG (1/5000 dilution; Sigma) for 1 h with gentle agitation, then washed in 0.02% BSA in PBS for 15 min before adding the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT; Sigma).

RESULTS

DEB in mitochondrial extracts

To determine whether mammalian mitochondrial extracts possessed DEB activity, we used EMSA. As depicted in Figure 1A (lane 1), free f394 DNA probe migrated to the bottom of a polyacrylamide gel. When probe f394 (0.2 ng) was incubated with mitochondrial protein extract in the presence of 120 ng of non-radioactive circular DNA, we detected a band of radioactivity with reduced mobility relative to the free probe (Fig. 1A, lane 2). This retarded band was not present in a parallel experiment in which 20 ng of unlabeled linear DNA was included in the reaction (Fig. 1A, lane 3). This latter finding indicates that the band with reduced mobility seen in Figure 1A (lane 2) resulted from the interaction of f394 and a protein that bound specifically to DNA ends.

To confirm the specificity of this mitochondrial DEB factor for DNA ends, we conducted an EMSA in which increasing amounts of either circular or linear DNA were added to a reaction containing radioactive probe f394 and mitochondrial protein extract. The amount of gel-shifted radioactivity present in each reaction was quantitated using phosphorimager analysis and this activity normalized to that detected in a standard reaction (see legend to Fig. 1B). As Figure 1B indicates, we observed a concentration-dependent decrease in amount of DEB activity as increasing amounts of unlabeled linear DNA were included in the reaction. Inclusion of 14 ng of unlabeled linear DNA in the incubation reaction reduced by 60% the amount of probe displaying reduced electrophoretic mobility (Fig. 1B). In contrast, inclusion of an additional 120 ng of circular unlabeled DNA resulted in only a 6% reduction in this value. (Note that the standard reaction contained 100 ng of circular unlabeled DNA. The amounts of added competitor DNA noted in Fig. 1B indicate the amount of DNA present in excess of this 100 ng of circular DNA.) We saw no further reduction in the amount of probe that displayed a gel shift when 300 ng of circular DNA was added to the reaction (data not shown). This experiment clearly demonstrates that the ability of a DNA to compete for the mitochondrial DEB activity is not dependent upon its sequence, but rather whether it possesses DNA ends. We further confirmed this finding by performing an EMSA experiment in which equal amounts of linear ϕX174 or $pREP_4$ DNA were used to compete for DEB activity. The presence of 100 ng of linearized ϕX174 or $pREP_4$ DNA reduced by 85 and 86%, respectively, the amount of f394 probe bound by the mitochondrial DEB activity (data not shown).

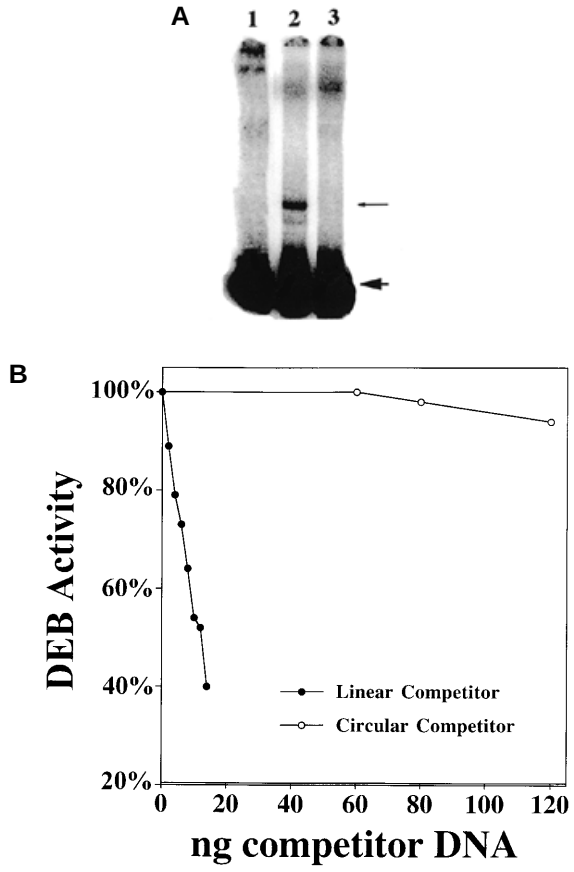

Figure 1. Mammalian mitochondrial extracts possess DEB activity. (**A**) Standard ESMAs were performed as described in Materials and Methods with specific changes as noted below. Lane 1, 0.2 ng probe f394 alone; lane 2, 0.2 ng probe f394, 120 ng circular competitor DNA and 2.4 µg mitochondrial extract; lane 3, 0.2 ng probe f394, 100 ng circular competitor DNA, 20 ng linear replicative form ϕX174 competitor DNA (see Materials and Methods for details on the competitor DNA used). The bold arrowhead indicates the position of the free f394 probe, while the thin arrowhead indicates the position of the DEB–probe complex. (**B**) A standard EMSA was performed [see (A), lane 2], as was an additional series of EMSAs in which increasing amounts of linear (filled circles) or circular (open circles) ϕX174 competitor DNA were included in the reaction. Following PAGE the gel was subjected to phosphorimager analysis. The band intensity of the shifted probe DNA was quantified using the program IPLab Gel. 100% DEB activity is defined as the value obtained from the standard reaction [see (A), lane 2]. The data are presented as the percent DEB activity in each reaction relative to that detected in the standard reaction. (Note that the amount of linear DNA added indicates the amount of competitor DNA added in addition to the 100 ng present in the standard reaction.)

Specificity of mitochondrial DEB activity for different DNA ends

We performed a series of competition experiments to determine the specificity of the mitochondrial DEB factor for different types of DNA ends. DEB experiments were performed as described above. Each reaction contained 0.2 ng of radioactive f394 probe and 250 ng of circular competitor DNA. In parallel experiments, 100 ng of linear DNA containing a 5'-overhang, a 3'-overhang or blunt ends were included. Each experiment was performed in triplicate and the ability of each DNA substrate to compete with the radioactive probe for the DEB activity was determined. As Figure 2 indicates, addition of any of the three types of linear DNA molecules reduced by ~75% the amount of radioactivity present in the shifted band. Statistical analysis of this data revealed that there was no significant difference in the ability of these substrates to compete for binding ($P > 0.05$). We therefore conclude that the DEB activity binds 5'-overhang, 3'-overhang and blunt-ended DNA ends with essentially equivalent affinity.

Affinity of the mitochondrial DEB protein for cohesive DNA ends

The results presented above indicate the presence of DEB activity in mitochondrial extracts. We measured the affinity of

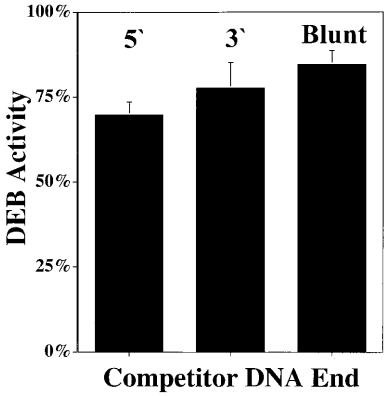

Figure 2. The mitochondrial DEB activity recognizes a broad range of DNA end substrates. Standard EMSAs were conducted in the presence of 1.6 μg crude rat liver mitochondrial extract. Each reaction also contained 100 ng circular and 100 ng of linear ϕX174 competitor DNA that had either a 3'-overhang, a 5'-overhang or blunt DNA ends as indicated. The percent DEB activity present under these various conditions was calculated as described in the legend to Figure 1B. The averages and standard errors were calculated from three independent experiments.

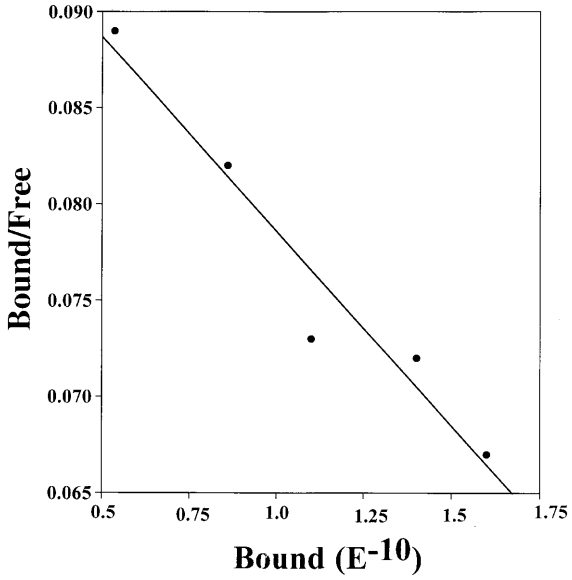

Figure 3. The mitochondrial DEB factor has a high affinity for DNA ends. A series of EMSA experiments was performed using increasing amounts of 5'-overhang linear ϕX174 competitor DNA. Phosphorimager analysis was used to measure the amount of bound (gel shifted) and free radioactivity present for each condition and a Scatchard analysis was performed (32).

this DEB factor for DNA ends by Scatchard analysis (32). This analysis revealed that the mitochondrial DEB factor bound linear DNA with a dissociation constant of 5 nM (Fig. 3). This value is very similar to that previously reported for Ku binding to double-stranded linear DNA (33,34).

Mitochondrial DEB activity is present in cells that lack nuclear DEB activity

The results presented above indicate that the mitochondrial DEB activity is functionally similar to Ku, a DEB protein that plays an essential role in nuclear DNA double-strand break repair (DSBR). It was therefore conceivable that Ku could be responsible for the DEB activity present in the mitochondrial extracts. For example, it was possible that the mitochondrial protein extracts described above were contaminated with nuclear proteins. If this were the case, one would expect that DEB activity measured as a function of total protein concentration would be significantly decreased in mitochondrial extracts relative to nuclear extracts. To test this, we measured nuclear and mitochondrial DEB activity using a standard EMSA followed by quantification of the shifted probe f394 using the program IPLab Gel. We observed that at all protein concentrations tested, the amounts of DEB activity in nuclear and mitochondrial extracts were similar (data not shown). The minimal concentration of protein extract needed to detect DEB activity was also similar among nuclear and mitochondrial extracts, indicating that the mitochondrial DEB activity is roughly equivalent in potency to that found in the nucleus. These observations strongly suggest that the mitochondrial activity is not dependent on the presence of contaminating nuclear proteins.

To conclusively rule out the possibility that contaminating Ku is responsible for the observed mitochondrial DEB activity, we prepared mitochondrial and nuclear protein extracts from

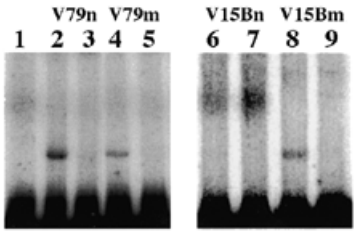

Figure 4. Mitochondrial DEB activity is present in cells that lack nuclear DEB activity. DEB activity was measured using standard EMSAs. Lane 1, free probe f394; lanes 2 and 3, 0.5 μg of V79 nuclear extract (V79n); lanes 4 and 5, 0.5 μg of V79 mitochondrial extract (V79m); lanes 6 and 7, 0.5 μg of XR-V15B nuclear extract (V15Bn); lanes 8 and 9, 0.5 μg of XR-V15B mitochondrial extract (V15Bm). Aliquots of 60 ng of linear ϕX174 competitor DNA were also included in lanes 3, 5, 7 and 9.

the Chinese hamster cell lines V79 and XR-V15B. The XR-V15B cell line (35) harbors a deletion in the gene encoding the Ku86 protein and was previously shown to lack nuclear DEB activity (20,23). Figure 4 confirms that whereas nuclear extracts prepared from V79 cells have DEB activity (V79n; lanes 2 and 3), XR-V15B nuclear extracts do not (V15Bn; lanes 6 and 7). In contrast, mitochondrial extracts prepared from V79 (V79m; lanes 4 and 5) and XR-V15B cells (V15Bm; lanes 8 and 9) have equivalent levels of DEB activity. These data rule out the possibility that mitochondrial DEB activity results from contamination of the extract by Ku.

The mitochondrial DEB factor is structurally related to Ku70/86

Although we ruled out the possibility that mitochondrial DEB activity resulted from contamination by Ku, the striking functional similarity led us to investigate whether antibodies to Ku would cross-react with the mitochondrial DEB factor. A 10% SDS–PAGE was prepared and molded to a comb that generated wells of either 1 or 3 cm in width. An aliquot of 5 μg of HT1080 nuclear protein extract was loaded in duplicate 1 cm wells, one on either side of a 3 cm well. An aliquot of 15 μg of HT1080 mitochondrial extract was loaded into the 3 cm well. Following electrophoretic transfer to nitrocellulose membrane, the membrane was cut length-wise into three strips. The three cuts were made to permit us to perform western blot analysis on nuclear and mitochondrial extracts using either or both anti-Ku70 and anti-Ku86 antisera. Western blot analysis of human nuclear (lanes 1 and 3) and mitochondrial (lane 2) protein extracts using anti-Ku polyclonal antisera showed that both anti-Ku70 and anti-Ku86 antisera cross-reacted with mitochondrial proteins (Fig. 5). As expected, nuclear proteins of 70 and 86 kDa were recognized by the anti-Ku70 and anti-Ku86 antisera, respectively (Fig. 5, lanes 1 and 3). These same antisera also cross-reacted with mitochondrial proteins of 70 and 68 kDa, respectively (Fig. 5, lane 2). (When we initially conducted western blot analysis of mitochondrial protein extract, both anti-Ku70 and anti-Ku86 antisera seemed to cross-react with a single protein; however, the experiment described above clearly indicates that anti-Ku70 antiserum cross-reacts with one mitochondrial protein and anti-Ku86 antiserum cross-reacts with another mitochondrial protein.)

We next wanted to test the hypothesis that the proteins recognized by anti-Ku antisera were also responsible for the mitochondrial DEB activity. To do this, we incubated radioactive probe f394 with circular competitor DNA and mitochondrial extract for 30 min at 14°C. After the 30 min incubation, anti-Ku70 antiserum, anti-Ku86 antiserum or control rabbit IgG was added to the EMSA reaction mixture, which was then incubated for an additional 1 h at 14°C. Methods similar to these have been successfully employed by others to identify proteins involved in EMSA experiments (36,37). As seen in Figure 6A and B, addition of anti-Ku70 and anti-Ku86 antisera, but not of control rabbit IgG, resulted in a 'supershift' of the DEB complex. In Figure 6A, free probe (lane 1) incubated with nuclear and mitochondrial extract in the presence of circular DNA formed bands with retarded mobility (lanes 2 and 4, respectively). In parallel experiments, nuclear and mitochondrial extracts were supershifted by 10 μg of anti-Ku70 antiserum (lanes 3 and 5, respectively) (the gel position of the supershifted DEB complex was dependent on the concentration of anti-Ku70 antiserum used; data not shown). Rabbit IgG (10 μg) did not supershift the DEB complex (lane 6). Including linear competitor DNA in the reaction abolished DEB to the radioactive probe f394 (lane 7). Similar experiments were performed using anti-Ku86 antiserum, as shown in Figure 6B. Again, the mobility of free probe (lane 1) was retarded by inclusion of nuclear or mitochondrial extract in the presence of circular competitor DNA (lanes 2 and 4, respectively). The nuclear and mitochondrial DEB complexes were then supershifted by 16 μg of anti-Ku86 antiserum (lanes 3 and 5, respectively), but not by 16 μg of rabbit IgG (lane 6). As lane 7 indicates, the DEB complex was not seen when excess linear competitor DNA was included in the reaction.

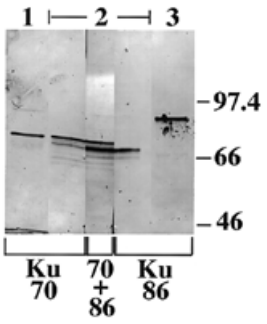

Figure 5. Anti-Ku70 and anti-Ku86 antisera cross-react with proteins in the mammalian mitochondria. Aliquots of 5 μg of duplicate samples of nuclear protein extract from human HT1080 cells were loaded in lanes 1 and 3. An aliquot of 15 μg of mitochondrial protein extract from these same cells was loaded in a single larger well (lane 2). Western blot analysis was performed as indicated using anti-Ku70, a mixture of anti-Ku70 and anti-Ku86 or anti-Ku86 polyclonal antisera (see Materials and Methods). The positions of marker proteins and their molecular weights are indicated (in kDa).

Analysis of Figure 6 indicates that the mobility of the mitochondrial DEB complex appeared to be greater than the mobility of the nuclear DEB complex (compare Fig. 6A, lanes 2 and 4). To confirm this finding, an additional EMSA experiment was performed on human nuclear and mitochondrial extracts using a smaller (94 bp) DNA fragment as a probe. Again, we observed that the mitochondrial DEB complex migrated faster than did the nuclear DEB complex (data not shown). Interestingly, this difference in mobility of the nuclear and mitochondrial DEB complexes was observed only in human extracts. No difference was detected between the mobility of nuclear and mitochondrial DEB complexes prepared from hamster cells using either f394 (Fig. 4) or the smaller 94 bp probe (data not shown).

Inspection of Figure 6 also revealed the presence of multiple gel-shifted bands in EMSA experiments performed using both nuclear and mitochondrial extract (Fig. 6B, lanes 2 and 6). It has been shown that the purified Ku heterodimer is capable of forming multiple gel-shift complexes. These complexes result from the binding of multiple Ku heterodimers to the probe and their formation is dependent upon Ku protein concentration and probe length (38). To determine if the mitochondrial DEB complex formed multiple gel shift bands we conducted an EMSA where the concentration of mitochondrial protein was increased from 0 to 0.4 μg. As Figure 7 reveals, as the amount of mitochondrial protein increased formation of multiple gel-shift complexes was observed. This experiment further reveals the functional similarities between nuclear and mitochondrial DEB activities.

DISCUSSION

The results presented above demonstrate that mammalian mitochondria possess DEB activity. We provide compelling evidence that this activity is not due to contamination of mitochondrial extract by nuclear proteins. First, the level of DEB activity in both the nuclear and mitochondrial protein

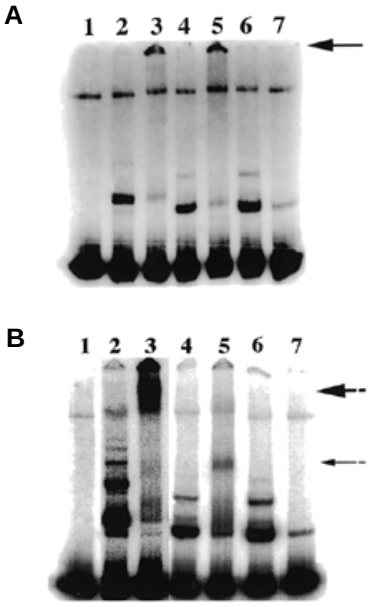

Figure 6. Anti-Ku70 and anti-Ku86 antisera 'supershift' DEB activity. (**A**) Standard EMSAs were performed with changes as indicated below. Lane 1, free probe f394 alone; lanes 2 and 3, 0.35 μg of nuclear protein extract from human HT1080 cells; lanes 4–7, 0.35 μg of mitochondrial protein extract from these same cells. Aliquots of 10 μg of anti-Ku70 antiserum were included in lanes 3 and 5. An aliquot of 10 μg of rabbit IgG antibody was included in lane 6. An aliquot of 200 ng of linear $pREP_4$ competitor DNA was included in lane 7. The arrow indicates the location of the Ku70-mediated supershift of nuclear and mitochondrial DEB activities in lanes 3 and 5 respectively. (**B**) Identical to the conditions in (A) with the following changes. Lane 1, free probe f394 alone; lanes 2 and 3, 0.35 μg of nuclear protein extract from HT1080 cells; lanes 4–7, 0.35 μg of mitochondrial protein extract from HT1080 cells. Aliquots of 16 μg of anti-Ku86 antiserum were included in lanes 3 and 5. An aliquot of 16 μg of rabbit IgG antibody was included in lane 6. An aliquot of 200 ng of linear $pREP_4$ competitor DNA was included in lane 7. The large arrow indicates the location of the Ku86-mediated supershift of nuclear DEB activity in lane 3. The small arrow indicates the location of the Ku86-mediated supershift of mitochondrial DEB activity in lane 5.

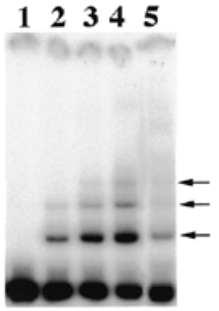

Figure 7. Mitochondrial DEB activity forms multiple gel shift bands as a function of protein concentration. Lane 1, free probe f394; lanes 2–4, 0.1, 0.2 and 0.4 μg HT1080 mitochondrial protein extract, respectively; lane 5, 0.4 μg HT1080 mitochondrial protein extract in the presence of 100 ng linear $pREP_4$ competitor DNA. Arrows indicate positions of gel-shifted bands due to mitochondrial DEB activity.

extracts was similar. In fact, the mitochondrial extract often had more DEB activity per unit of protein than did the nuclear extract. More convincingly, we have shown that wild-type levels of mitochondrial DEB activity are present in mitochondrial proteins extracts prepared from the cell line XR-V15B. These data are inconsistent with the hypothesis that trace amounts of nuclear protein in the mitochondrial extract are responsible for mitochondrial DEB activity.

Nevertheless, the mitochondrial DEB activity is functionally very similar to nuclear Ku-mediated DEB activity. First, both the nuclear and mitochondrial DEB activities are highly specific for linear DNA. Second, both the nuclear and mitochondrial DEB activities bind with similar affinity to different types of DNA ends. Third, both the nuclear and mitochondrial DEB activities have an affinity for DNA ends in the low nanomolar range. Fourth, both the nuclear and mitochondrial DEB activities form multiple gel shifted bands in EMSAs. Formation of these multiple gel-shift complexes is dependent on the protein concentration.

In addition to these functional similarities, the human nuclear and mitochondrial DEB activities appear to be structurally similar as well, since antisera that recognize each of the components of the nuclear Ku DEB activity are able to 'supershift' the mitochondrial DEB complex in EMSA experiments. Anti-Ku70 antiserum had a qualitatively similar effect on supershifting both nuclear and mitochondrial DEB proteins. In contrast, while anti-Ku86 antiserum cross-reacted with the mitochondrial DEB activity, it clearly reacted more strongly with the nuclear DEB complex. Interestingly, human nuclear and mitochondrial extracts both contained a 70 kDa protein that cross-reacted with anti-Ku70 antiserum. In contrast, western blots performed using anti-Ku86 antiserum revealed the presence of proteins of 86 and 68 kDa, respectively, in these same two extracts. It is possible, although not yet proven, that the difference in size of these anti-Ku86 cross-reacting proteins provides an explanation for the difference between the mobility of the nuclear and mitiochondrial DEB complexes. We have been unable to perform similar analysis on hamster nuclear and mitochondrial DEB complexes due to poor cross-reactivity of these anti-Ku antisera with hamster proteins.

Based on the results summarized above, one can imagine at least three possible sources for the mitochondrial DEB activity. The first possibility is that mitochondrial DEB activity results from alternative splicing of either or both of the nuclear Ku70 and Ku86 genes. The second possibility is that post-translational modification of either or both of the Ku70 and Ku86 proteins generate novel molecules. In either event, the novel forms of these proteins would presumably be targeted to the mitochondrial compartment. The third possibility is that one or more novel nuclear genes encode the mitochondrial DEB activity. Obviously these different possibilities are not mutually exclusive, since the multiple components of the mitochondrial DEB activity could arise from more than one of these sources.

The XR-V15B cell-line is characterized by a 46 amino acid deletion in the Ku86 mRNA (20). The presence of mitochondrial DEB activity in this cell line, however, does not permit us to distinguish between these three possibilities. While the nuclear Ku86 protein of XR-V15B cells is defective, either alternative splicing or post-translational modification could

generate a mitochondrial Ku86 isoform that has DEB activity. Alternatively, of course, it is possible that the mitochondrial Ku86-like activity found in these cells is due to a novel gene that encodes a Ku86 homolog. Clearly this cell line does not provide any insight into the source of the mitochondrial protein that cross-reacts with Ku70 antiserum. Thus, additional experimentation will be required to determine the source of the mitochondrial DEB activity we have identified.

We have previously shown that mitochondrial protein extracts possess potent DNA end-joining activity (16). Furthermore, our recent data indicate that this activity is catalyzed by a mitochondria-specific form of DNA ligase III (8). Experiments performed using purified DNA ligase III reveal that this enzyme is nearly incapable of catalyzing DNA end-joining *in vitro* (25). Intriguingly, these investigators observed that when purified recombinant Ku heterodimer is present, there was a substantial (~30-fold) increase in end-joining activity of ligase III. Thus, the ability of mitochondrial protein extracts to catalyze efficient DNA end-joining *in vitro* (16) strongly suggests the presence of an activity functionally analogous to Ku. It is therefore possible that the mitochondrial DEB activity described herein is involved in DSBR in this organelle.

The existence of a mtDNA DSBR pathway is also consistent with the observation that mtDNA deletions accumulate in aged humans and other mammals. Molecular analysis reveals that these deletions span direct DNA sequence repeats (39–42). As our laboratory has previously demonstrated (16), DNA end-joining reactions catalyzed by mitochondrial protein extracts occasionally result in the generation of deletion-bearing molecules strikingly similar to those that arise in aged mammalian tissues. Thus, it is conceivable that the presence of deletion-bearing mtDNA in aged mammals results from the occasional mis-repair of mtDNA double-strand breaks. A number of observations raise the prospect that deficient mitochondrial DSBR could play a role in the generation of these deletions. For example, studies examining DSBR in yeast revealed that the absence of nuclear DEB activity is associated with faulty DNA end-joining (26,28). Under these conditions, the great majority of products of the end-joining reaction have suffered deletions that span direct DNA sequence repeats. In addition, it has been shown that end-joining in Ku86-deficient cells is imprecise, relative to wild-type cells (43). Based on these observations, we believe that the mitochondrial DEB activity we have described is involved in a pathway for DSBR similar to that observed in the nucleus.

ACKNOWLEDGEMENTS

We thank G. Chu and M. Zdzienicka, who generously provided the V79 and XR-V15B cell lines, and Dr Susan Critchlow, Wellcome/CRC Institute, UK, who provided polyclonal Ku70/80 antibody. We also thank members of the Campbell laboratory for helpful editorial comments. This work was supported in part by grants from the NIH (R29 CA61906), the American Heart Association (9601390) and the American Cancer Society (DHP-171). U.L. was supported by a fellowship from the American Heart Association.

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