Mitochondrial Endonuclease Activities Specific for Apurinic/Apyrimidinic Sites in DNA from Mouse Cells*

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Endonuclease activity which specifically cleaves baseless (apurinic/apyrimidinic (AP)) sites in supercoiled DNA has been purified from mitochondria of the mouse plasmacytoma cell line, MPC-11. Two variant forms separate upon purification; these have small but reproducible differences in catalytic and chromatographic properties, but similar physical properties. Both have a sedimentation coefficient of 4.0, corresponding to a molecular weight of 61,000 (assuming a globular configuration) and a peptide molecular weight of about 65,000 as determined by immunoblot analysis with antiserum raised against the major AP endonuclease from HeLa cells. Thus mitochondrial AP endonuclease appears to be a monomer of about 65 kDa, making it distinguishable from the major AP endonuclease of MPC-11 cells which, like those of other mammalian cells, appears to be a monomer of about 41 kDa. A possible 82-kDa precursor form was also detected by immunoblot analysis of a crude mitochondrial fraction. Mitochondrial AP endonuclease activity is greatly stimulated by divalent cations, has a pH optimum between 6.5 and 8.5, and cleaves the AP site by a class II mechanism to generate a 3' OH nucleotide residue. These properties resemble those of the major mammalian AP endonucleases but, unlike those enzymes, mitochondrial AP endonuclease activity is neither inhibited by adenine or NAD+ nor stimulated by Triton X-100. Since the mitochondrial activity generates active primer termini for DNA synthesis, it could function in base excision DNA repair; alternatively, it might have a role in eliminating damaged mitochondrial genomes from the gene pool.

It has been estimated that roughly 10,000 purines and 200 pyrimidines are lost from a mammalian cell genome by spontaneous hydrolysis/day (1). In addition, baseless (AP)1 sites are produced in DNA by the action of various chemicals and radiation (2) and by DNA glycosylases which remove particular abnormal bases from DNA (3) for a review, see Ref. 4). AP endonucleases, which cleave DNA adjacent to baseless sites, are ubiquitous in living organisms, having been detected in bacteria (5, 6), plants (7, 8), Drosophila (9), and various mammalian cell types (10-13). Human AP endonucleases have been described from placenta (14), lymphoblasts (15), fibroblasts (16), and HeLa cells (17), and enzymes from mouse plasmacytoma (11), HeLa (17), and calf thymus (13) have been purified to apparent homogeneity as judged by gel electrophoresis. The N-terminal amino acid sequence of the latter enzyme has been determined.

AP endonucleases have been classified according to the position of the phosphodiester bond cleavage relative to the AP site (18). Class I enzymes appear to act by β-elimination, leaving an unsaturated baseless sugar at the 3' terminus of the nick, whereas class II enzymes act by hydrolysis leaving a deoxyribose phosphate at the 5' terminus; the two enzymes can act in concert in vitro to remove a sugar phosphate to produce a gap of 1 nucleotide (19). Alternatively, the repair of AP sites can occur by the action of an AP endonuclease plus an appropriate exonuclease.

Mammalian mitochondria contain genetic material in the form of multiple copies of a closed circular DNA molecule (20), but these organelles apparently lack nucleotide excision DNA repair and recombinational DNA repair (21). On the other hand, uracil DNA glycosylase, a base excision repair enzyme, has been detected in mitochondria (22-24), and AP endonuclease activity has been reported to be associated with crude mitochondrial (12) and chloroplast (25) extracts. In this paper, we report the purification and characterization of mitochondrial AP endonuclease activity from mouse MPC-11 plasmacytoma cells and compare it to the major extramitochondrial AP endonuclease from these cells (11).

EXPERIMENTAL PROCEDURES2

RESULTS

Association of AP Endonuclease Activity with Mitochondria—When mitochondria are purified by differential centrifugation and sedimentation through a sucrose step gradient, they are essentially free from cytoplasmic contamination (36) and lysosomal enzymes (29). When mitochondria were prepared in this manner, they contained about 1-3% of the total cellular AP endonuclease activity (see Miniprint, Table 1%), a value similar to that obtained by Thibodeau and Verly (12). After the intact mitochondria were further purified by sedimentation through a linear sucrose gradient, this AP endonuclease activity co-sedimented with fumarase, a mitochondrial marker enzyme, and no significant activity was detected near the top of the gradient, the expected position for soluble proteins (Fig. 1). In addition, a greater than 2-fold increase in AP endonuclease activity was observed when comparing

2 Portions of this paper (including "Experimental Procedures" and Table 1%) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from C. V. Mosby Co.

1 The abbreviations used are: AP, apurinic/apyrimidinic; dNMP, deoxynucleoside monophosphate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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assays of sonicated mitochondria with intact organelles (data not shown). These results suggest that the AP endonuclease activity detected in mitochondria is likely to be specifically associated with the mitochondria.

**Purification of Mitochondrial AP Endonuclease Activities**
To test whether a distinguishable AP endonuclease was indeed associated with mitochondria, the activity was purified (as described in the Miniprint). Two active fractions were resolved by phosphocellulose chromatography, and these activities, designated forms A and B, were each further purified by heparin-agarose chromatography. The heparin-agarose fractions were subjected to SDS-PAGE, and cross-reactivity was determined by immunoblot (Western)

**Physical Properties of Mitochondrial AP Endonuclease Activity**—Both forms sedimented through a linear sucrose gradient with a sedimentation coefficient of 4.0 S, and each had a minor fraction near 3.1 S. Assuming a globular protein conformation, these sedimentation coefficients would correspond to molecular weights of 61,000 and 41,000, respectively (37). When protein from peak fractions of each sucrose gradient was separated by SDS-PAGE and visualized by silver staining, several bands were present in the molecular weight range of 50,000–70,000.

Antibody raised against the major AP endonuclease of HeLa cells (17) cross-reacts with AP endonucleases from *Escherichia coli* and *Drosophila* (9). When cross-reaction with the mouse mitochondrial activities was tested by the ability of the antibody to precipitate the AP endonuclease activities in the presence of *Staphylococcus A* protein, there was significant cross-reactivity. Form A was precipitated with about 10% efficiency and form B with about 1% efficiency compared to the HeLa enzyme on a unit-by-unit basis (data not shown).

Since the antibody appeared to recognize the murine mitochondrial enzymes, the heparin-agarose mitochondrial AP endonuclease fractions were subjected to SDS-PAGE, and cross-reactivity was determined by immunoblot (Western) analysis. Cross-reacting proteins were detected at 66 ± 2 kDa for both forms A and B (data not shown due to faintness of bands). When an extract from unfractionated sonicated mitochondria was similarly tested, bands were seen with molecular masses corresponding to 65 ± 2 and 82 ± 2 kDa (Fig. 2A). Therefore, it appears that there are no significant physical differences between the two mouse mitochondrial enzyme forms and that each is active as a monomer of molecular weight near 65,000.

The unfractionated cytoplasmic/soluble fraction contained a triplet of immunoreactive peptides of molecular weight about 41,000 (Fig. 2, B and D). Faint immunoreactive bands corresponding to molecular weights of 65,000 and 82,000 were occasionally also present (Fig. 2B), possibly due to some mitochondrial lysis or to the presence of precursor forms. To be sure that the 82,000-dalton peptide from mitochondria was not reacting nonspecifically with the second antibody, the first antibody was omitted from the procedure and no bands were detected (Fig. 2C). When this same gel was then reblotted with the anti-AP endonuclease antibody, then again with the second antibody, bands were detected (Fig. 2D).

The larger 82-kDa protein may be a precursor protein which is processed to a 65-kDa protein for the mitochondria. The 41-kDa cytoplasmic form is the size of major AP endonucleases in HeLa cells (17) and calf thymus (13) as well as in mouse cells. The smaller 3.1 S shoulder of activity in the sucrose gradient might be due to subsequent proteolytic cleavage during purification of the mitochondrial activities.

**Mechanism of Cleavage**—AP endonucleases can be classified according to whether they generate an efficient primer for DNA synthesis after cleavage of the AP site. Both mitochondrial forms appear to cleave AP sites by the same mechanism as does the major AP endonuclease of all mammalian cells, i.e. the generation of a 3'-OH terminal nucleotide which can prime synthesis by *Escherichia coli* DNA polymerase I (Fig. 3A). *E. coli* endonuclease III (used as a control) is a class I enzyme which leaves an unsaturated sugar phosphate at the 3' terminus and therefore does not efficiently prime DNA synthesis (19). A further test of a class II AP endonuclease is
its ability to act on termini generated by endonuclease III so as to remove the baseless sugar phosphate from the 3'-end and generate an effective primer terminus. The mitochondrial isozymes also can enhance DNA synthesis from such termini (Fig. 3B).

Substrate Specificity—The mitochondrial endonuclease activities were specific for abasic DNA sites. No significant endonucleolytic activity was detected on undamaged or UV-irradiated supercoiled PM2 DNA (Table 1). When measuring the rate of nicking of partially depurinated PM2 DNA which had been relaxed by the action of rat liver topoisomerase I, forms A and B were one-third and one-tenth as active as upon the analogous supercoiled substrate, respectively. Both forms were free of detectable exonuclease activity as measured by release of acid-soluble material from heat-denatured E. coli DNA (Table 1).

Co-factor Requirements—The mitochondrial AP endonuclease forms A and B are stimulated as much as 20- and 10-fold, respectively, by divalent cations. Optimal activity is with 1 mM MgCl₂ for form A and 5 mM MgCl₂ for form B. Mn²⁺ and Co²⁺ are equally effective, and Zn²⁺ also stimulates about half as effectively; Ca²⁺ has little effect on enzyme activity.

Form A has a broad pH optimum between 6.5 and 9.0 with highest activity in 50 mM Tris-HCl (pH 8.0). Form B has a narrower pH optimum between 6.5 and 7.5 with highest activity in 50 mM imidazole HCl at pH 7.0.

Form A has optimal activity in 20 mM NaCl or KCl, whereas form B is more active without salt, though this difference may be due to the higher Mg²⁺ concentration used in assays of the latter. The major HeLa AP endonuclease activity is stimulated greatly by Triton X-100 (17), but no such effect was observed with the mitochondrial activities. The major AP endonucleases from HeLa cells and calf thymus are also inhibited by adenine and several adenine derivatives (13, 17); however, the mitochondrial activities are not inhibited by adenine or NAD⁺.

DISCUSSION

The extents of purification of the two mitochondrial AP endonuclease activities are not large (about 100-fold for form A and 50-fold for form B), partly because purification was from fractionated mitochondria and partly due to the instability of the enzymes during purification. The two forms have similar physical and catalytic properties, but they differ in their optimal reaction conditions and purification characteristics. Since they both appear to have monomeric molecular weights of about 65,000, they are not related by extensive proteolysis. It is more likely that the differences are caused by different protein modifications or by very limited proteolytic action. Multiple species of AP endonuclease have been detected in human placenta (14), and post-translational modification of the major human AP endonuclease which alters the catalytic properties has been demonstrated (38).

The major AP endonucleases from HeLa cells (17), calf thymus (13), and the nuclei of mouse plasmacytoma MPC-11 cells (11) all have similar enzymatic and physical properties. Although the mouse mitochondrial activity has similar catalytic properties compared with the major nuclear forms, the molecular mass of the mitochondrial activity is near 65 kDa compared to 41 kDa for the extracellular mouse endonuclease. The latter value is the same as the molecular weights reported for the enzymes from HeLa (17) and calf thymus (13) but differs from the previously reported molecular weight of 28,000 for the mouse enzyme as determined by gel filtration (11). This difference could reflect less proteolysis, but more likely it may be due to the different techniques used for
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Fig. 3. Mechanism of action of the mitochondrial AP endonuclease activities. A, reaction mixtures, as described in the Miniprint, contained 16.5 units of mitochondrial enzyme form A (○—○); 11.8 units of mitochondrial enzyme form B (●—●); 5.1 units of the class II human fibroblast AP endonuclease II (■—■); 3.3 units of the class I E. coli endonuclease III (●—●); or no enzyme (○—○). Reactions with the mitochondrial isozymes were incubated at 37°C for 15 min, whereas the other assays were incubated for 30 min. The incised DNA was subsequently used as substrate for DNA synthesis with 0.05 units of E. coli DNA polymerase I and the total molecules of dNMP incorporated per incision determined. The number of incisions/PM2 genome, as determined by endonuclease assay, is given in parentheses. B, reaction mixtures were prepared as described in the Miniprint containing 4.7 units of mitochondrial enzyme form A (○—○); 7.9 units of E. coli endonuclease III (●—●); 4.7 units of enzyme form A plus 7.8 units of E. coli endonuclease III (■—■); or no endonuclease (○—○). The first reaction was incubated at 37°C for 15 min, while the others were incubated for 30 min. DNA synthesis was carried out as described in part A. Reactions with mitochondrial enzyme form B gave similar results to those with form A (data not shown).

Table I

Substrate specificity of mitochondrial AP endonucleases

Nuclease activities were measured in 50 mM Tris-HCl (pH 8.2), 1 mM MgCl₂ (isozyme A) and in 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂ (isozyme B). The various substrates were prepared as described in the Miniprint. Endonuclease assays contained 2.4 nmol of PM2 DNA for supercoiled substrates and 1.8 nmol for the relaxed substrate.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Substrate</th>
<th>Isozyme I</th>
<th>Isozyme II</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP endonuclease (standard assay)</td>
<td>Supercoiled depurinated PM2 DNA</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Relaxed depurinated PM2 DNA</td>
<td>29</td>
<td>9.1</td>
</tr>
<tr>
<td>&quot;UV endonuclease&quot;</td>
<td>Supercoiled UV-irradiated PM2 DNA</td>
<td>2.4</td>
<td>0.7</td>
</tr>
<tr>
<td>Exonuclease</td>
<td>Single-stranded E. coli DNA</td>
<td>None</td>
<td>None</td>
</tr>
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</table>

measuring molecular weight, since a difference in apparent molecular weight was detected for the HeLa AP endonuclease when comparing gel filtration to SDS-PAGE data (17).

Several mammalian mitochondrial genomes have been sequenced, and all potential open reading frames have been assigned to proteins involved in mitochondrial structure or the respiratory chain. Therefore, all of the proteins responsible for the replication and maintenance of the mitochondrial genome must be encoded by the nuclear genome. In yeast, the nuclear gene encoding the mitochondrial RNA polymerase has been identified and appears to be separate from the genes encoding the nuclear RNA polymerase (39). Proteins can be marked for mitochondrial localization by the presence of a particular amino acid sequence at or near the N terminus (40). It is possible that the mitochondrial AP endonucleases are directed to the mitochondria from the cytoplasm by this mechanism, although it is unlikely that the large difference in molecular weight between the nuclear and mitochondrial enzymes is for an N-terminal signal sequence. An alternative mechanism for directing proteins to specific subcellular locations is specific proteolytic cleavage of a large, possibly inactive, precursor protein to generate different active polypeptide fragments, which act in different subcellular locations. An example of this is the production of nuclear, lysosomal, and mitochondrial forms of a nuclease from a common precursor in Neurospora crassa (41). Mouse mitochondria contain a similar nuclease activity (29). If this were also the case for AP endonucleases, it might account for the larger 82-kDa peptide observed by immunoblot analysis of unfractionated mitochondrial extracts. Peptide sequencing and/or gene cloning should ultimately resolve these issues.

Since mammalian mitochondria do not carry out significant amounts of nucleotide excision repair or postreplication recombinational repair, it has been suggested that mitochondria do not repair DNA damage but instead destroy damaged templates and replicate only the undamaged copies. Such elimination might explain the low rate of mutation in mitochondrial DNA of somatic cells. This scheme would be reasonable in the case of rarely occurring lesions, but for a frequently occurring lesion such as a uracil residue or an AP site, it probably would be more efficient to repair the lesion than degrade the genome. The presence in mitochondria of...
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AP endonuclease activities in addition to uracil DNA glycosylase (22–24) and several glycosylases which recognize oxyradical damage3 suggests that DNA base excision repair functions in these organelles. Of course it still could be argued that these enzymes are not involved in DNA repair but function instead to initiate degradation of damaged templates. However, the mitochondrial AP endonucleases, like the major cellular AP endonucleases, cleave AP sites by a class II mechanism and generate efficient primers for DNA synthesis. For a supercoiled DNA substrate, and the mitochondrial genome is supercoiled in vivo. Therefore, these AP endonucleases prefer an undamaged to a nicked genome produced either by DNA damage or by the action of another nuclease. The question of the existence of DNA repair in mitochondria will probably be resolved by isolation and characterization of AP endonuclease activities in addition to uracil DNA glycosylase.

Acknowledgments—We thank B. Johnson for her expert help with tissue culture and Caroline Kane for antibody to HeLa AP endonuclease.

REFERENCES

SUPPLEMENTAL MATERIAL

Mitochondrial Endonuclease Activities Specific for Adenosine/Thymidine in DNA from Mouse Cells

A. E. Tomkinson, R. T. Bonk, and S. Linn, unpublished data.

Mitochondrial AP Endonuclease Activities

**Table 10**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Assay Conditions</th>
<th>Volume</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>10 mg</td>
<td>20 ml</td>
<td>1.44 x 10^6</td>
<td>1.11 x 10^7</td>
<td></td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>10 mg</td>
<td>1 ml</td>
<td>8.69 x 10^6</td>
<td>4.76 x 10^7</td>
<td></td>
</tr>
<tr>
<td>Oxygen</td>
<td>10 mg</td>
<td>2 ml</td>
<td>1.73 x 10^6</td>
<td>1.05 x 10^7</td>
<td></td>
</tr>
<tr>
<td>Phosphate</td>
<td>10 mg</td>
<td>5 ml</td>
<td>3.09 x 10^6</td>
<td>3.09 x 10^7</td>
<td></td>
</tr>
<tr>
<td>Phosphate + NaCl</td>
<td>10 mg</td>
<td>2 ml</td>
<td>7.54 x 10^6</td>
<td>1.31 x 10^7</td>
<td></td>
</tr>
</tbody>
</table>

For stimulation of AP-endonuclease activity, the mitochondrial matrix was isolated from rat liver mitochondria by the method of Schachter et al. (1967). The mitochondria were suspended in 0.25 M sucrose, 10 mM Tris-Cl, pH 7.4, 1 mM EDTA, and 20 μM dithiothreitol (DTT). The suspension was incubated at 37°C for 30 min. Then the suspension was centrifuged at 100,000 x g for 30 min to remove the supernatant. The supernatant was used as the assay sample.